Developmental Biology 455 (2019) 100–111

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Modulating eIF6 levels unveils the role of translation in ecdysone biosynthesis during *Drosophila* development

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ARTICLE INFO

Keywords:
eIF6
Ecdysone
20HE
Translation
Drosophila development

ABSTRACT

During development, ribosome biogenesis and translation reach peak activities, due to impetuous cell proliferation. Current models predict that protein synthesis elevation is controlled by transcription factors and signalling pathways. Developmental models addressing translation factors overexpression effects are lacking. Eukaryotic Initiation Factor 6 (eIF6) is necessary for ribosome biogenesis and efficient translation. *eIF6* is a single gene, conserved from yeasts to mammals, suggesting a tight regulation need. We generated a *Drosophila melanogaster* model of eIF6 upregulation, leading to a boost in general translation and the shut-down of the ecdysone biosynthetic pathway. Indeed, translation modulation in S2 cells showed that translational rate and ecdysone biosynthesis are inversely correlated. *In vivo*, eIF6-driven alterations delayed Programmed Cell Death (PCD), resulting in aberrant phenotypes, partially rescued by ecdysone administration. Our data show that eIF6 triggers a translation program with far-reaching effects on metabolism and development, stressing the driving and central role of translation.

1. Introduction

During cell proliferation, ribosomal proteins (RPs) and eukaryotic Initiation Factors (eIFs) are necessary and in high demand for ribosome biogenesis and translation (Hershey et al., 2012; Kressler et al., 1999, 2017; Venema and Tollervey, 1999; Warner et al., 2001). Proteins involved in ribosome biogenesis do not usually have a role in the translational control and *vice versa* (Miluzio et al., 2009). However, the eukaryotic Initiation Factor 6 (eIF6) is remarkably unique (Brina et al., 2015a): a nuclear pool is essential for nucleolar maturation of the 60S large subunit (Gandin et al., 2008), while cytoplasmic eIF6 acts as a translation factor (Gandin et al., 2008). Mechanistically, eIF6 is an anti-association factor: by binding 60S subunit, eIF6 prevents its premature joining with a 40S not loaded with the pre-initiation complex. Release of eIF6 is then mandatory for the formation of an active 80S (Ceci et al., 2003). The dual action of eIF6 in ribosome biogenesis and translation suggests that it may act as a master gene regulating ribosomal efficiency. Remarkably, point mutations of eIF6 can revert the lethal phenotype of ribosome biogenesis factors such as SBDS (Menne et al., 2007) and eFL1p (Wong et al., 2011). eIF6 is highly conserved in yeast, fruit fly and humans (Biffo et al., 1997). During evolution, the *eIF6* gene has not been subjected to gene duplication. Despite its ubiquitous role, eIF6 levels are tightly regulated *in vivo*, showing considerable variability of expression among different tissues (Donadini et al., 2001). Importantly, high levels of eIF6 or hyperphosphorylated eIF6 are observed in some cancers (Miluzio et al., 2015; Sanvito et al., 1999). eIF6 is rate limiting for tumor onset and progression in mice (Miluzio et al., 2011). In addition, *eIF6* amplification is observed in luminal breast cancer patients

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https://doi.org/10.1016/j.ydbio.2019.05.013
Received 5 December 2018; Received in revised form 1 May 2019; Accepted 28 May 2019
Available online 5 July 2019

(Gatza et al., 2014) and affects cancer cell metastatization (Benelli et al., 2012; Pinzaglia et al., 2015). It has been recently demonstrated that eIF6 acts at the translational level through the regulation of metabolism: in mammals, eIF6 translation activity increases fatty acid synthesis and glycolysis through the translation of transcription factors such as CEBP/β, ATF4 and CEBP/δ containing G/C rich or uORF sequences in their 5′UTR (Brina et al., 2015b; Miluzio et al., 2016).

However, whether eIF6 overexpression *per se* can change a transcriptional program in the absence of other genetic lesions is unknown.

Ecdysone is the primary steroid hormone in insects: during fly development, it is produced as a precursor, ecdysone (E) in the prothoracic gland (PG). Biosynthesis starts from cholesterol and, after several enzymatic steps, it is secreted in the haemolymph. Target tissues convert ecdysone into the active form, the 20-hydroxyecdysone (20HE) (Gilbert et al., 2002). The binding of 20HE with its receptor is responsible for a transcriptional cascade that triggers metamorphosis (Yamanaka et al., 2013). Pulses of 20HE regulate several processes such as cell proliferation, differentiation and cell death (Yamanaka et al., 2013; Champlin and Truman, 1998a, 1998b; Herboso et al., 2015; Lee et al., 2000; Nicolson et al., 2015).

To determine the effects of eIF6 high levels *in vivo*, we took advantage of *Drosophila melanogaster*, an ideal model to manipulate gene expression in a time- and tissue-dependent manner, using the GAL4/UAS system (Brand and Perrimon, 1993; del Valle Rodriguez et al., 2011). We reasoned that an overexpression approach could allow us to evaluate the effects of eIF6 increased activity in the context of an intact organism. To this end, we focused on the fly eye, an organ not essential for viability, whose development from epithelial primordia, the larval eye imaginal disc, is well known. The adult compound eye is a stunningly beautiful structure of approximately 800 identical units, called ommatidia (Kumar, 2012). Each ommatidium is composed of eight neuronal photoreceptors, four glial-like cone cells and pigment cells (Cagan and Reh, 2010; Ready et al., 1976).

By increasing eIF6 levels specifically in the eye, we found alterations in physiological apoptosis at the pupal stage, correlating with an increase in general translation.

We observed that increased levels of eIF6 are responsible for a reshaping of the eye transcriptome that revealed a coordinated down-regulation of the ecdysone biosynthetic pathway during the larval stage. This study provides the first *in vivo* evidence that an increase in translation, dependent on heightened eIF6 levels, may drive metabolic changes and a transcriptional rewiring in a developing organ.

2. Materials and methods

2.1. Genetics

Fly strains were maintained on standard cornmeal food at 18 °C. Genetic crosses were performed at 25 °C, with the exception of *GMRGAL4/+* and *GMR > eIF6*, performed at 18 °C. The following fly mutant stocks have been used: *GMRGAL4* was a gift from Manolis Fanto (King's College, London); *UAS-eIF6* was a gift from William J Brook (Alberta Children's Hospital, Calgary). The *eIF6* (GH08760) cDNA was obtained from the Berkeley Drosophila Genome project (Research Genetics) and sequenced for confirmation. The entire *eIF6* cDNA was cloned into the RI site of the pUAST (Ji et al., 2008). Lines obtained from the Bloomington Drosophila Stock Center (BDSC): *y[1] w[*]; P{w[+mC] = tubP-GAL4}LL7/TM3, Sb[1] Ser[1]* (5138); *spaGAL4* (26656) *P{w[+mC] = spa-GAL4.J}1, w[*]*; *54CGAL4* (27328) y*[1] w[*]; P{w[+m*] = GAL4}54C*; *w1118* (3605) *w[1118]*; *bxMS1096GAL4* (8860) w[1118] *P{w[+mW.hs] = GawB}Bx[MS1096]*.

2.2. Mosaic analysis

The *eIF6*^{k13214} mutant clones were created by *Flippase* (*FLP*) mediated mitotic recombination (Harrison and Perrimon, 1993). The *eIF6*^{k13214} *(P(w[+mC] = lacW) eIF6[k13214] ytr[k13214]) P* element allele was recombined onto the right arm of chromosome two with the homologous recombination site (FRT) at 42D using standard selection techniques. Briefly, to create the FRT *y*$^{+}$ *pwn, eIF6*^{k13214} chromosomes, *eIF6*^{k13214} was recombined onto the FRT chromosome originating from the *y; P42D pwn [1] P{y+}44B/CyO* parental stock. The *yellow*$^{+}$ *pwn eIF6*^{k13214}*G418* resistant flies were selected to create stocks for clonal analysis. Similarly, stocks used for generating unmarked *eIF6*^{k13214} clones were created by recombining *eIF6*^{k13214} with the 42D FRT chromosome using the *w [1118]; P42D P{Ubi-GFP}2R/CyO* parental line. Targeted mitotic wing clones were generated by crossing flies with *UAS-FLP*, the appropriate GAL4 driver and the suitable *42D* FRT second chromosome with the *42D* FRT *eIF6*^{k13214}. The *hs* induced *eIF6*^{k13214} mitotic clones were created by following standard techniques. Briefly, 24- and 48-h larvae with the appropriate genotypes were heat shocked for 1 h at 37 °C followed by incubation at 25 °C.

2.3. S2 cell culture

The *Drosophila* S2 cells (RRID: CVCL_TZ72) were grown in Schneider medium (Lonza, Basel, Switzerland, #04-351Q) supplemented with 10% Fetal Bovine Serum (FBS – #ECS0180L, Euroclone, Pero, Italy) and 5 mL of PSG 1X (100X composition: 10000 U/mL Penicillin, 10 mg/mL Streptomycin and 200 mM L-Glutamine in citrate buffer, (#G1146, Sigma, St. Louis, MO, USA), and maintained as a semi-adherent monolayer at standard culture conditions at 25 °C without CO_2. For protein synthesis measurement, S2 cells were treated at 65–70% confluence with 1 μM rapamycin (#R8781, Sigma) for 2 h or 1 μM insulin (#I0516, Sigma) for 12 h, both at 25 °C. For SUnSET assay, the medium was removed and replaced with fresh medium supplemented with 5 μg/mL puromycin (#A1113803, Thermofisher Scientific, Waltham, MA, USA) for 3 h, and treated according to (Schmidt et al., 2009).

2.4. RNA isolation and RNA sequencing

Total RNA was extracted with the mirVana™ isolation kit according to the manufacturer protocol (#AM 1560, ThermoFisher) from 10 eye imaginal discs (larval stage) or 10 retinae (pupal stage). The RNA quality was controlled with BioAnalyzer (Agilent, Santa Clara, CA, USA). Libraries for Illumina sequencing were constructed from 100 ng of total RNA with the Illumina TruSeq RNA Sample Preparation Kit v2 (Set A) (Illumina, San Diego, CA, USA). The generated libraries were loaded on to the cBot (Illumina) for clustering on a HiSeq Flow Cell v3. The flow cell was then sequenced using a HiScanSQ (Illumina). A paired-end (2 × 101) run was performed using the SBS Kit v3 (Illumina). Sequence deepness was at 35 million reads. For quantitative PCR, the same amount of RNA was retrotranscribed according to SuperScript™ III First-Strand Synthesis SuperMix manufacturer protocol (#18080400, LifeTechnologies, Carlsbad, CA, USA). For RNA-Seq validation, TaqMan probes specific for *eIF6* (Dm01844498_g1) and *rpl32* (Dm02151827_g1) were used, together with standard primers (*rpl32* Fwd CGGATCGATATGCTAAGCTGT, Rev CGACGCACTCYCYYGTCG; *shd* Fwd CGGGCTACTCGCTTAATGCAG, Rev AGCAGCACCACCTCCATTTC). Target mRNA quantification was performed by using ΔCt-method with *rpl32* RNA as an internal standard, performed on a StepOne Plus System (Applied Biosystems, Foster City, CA, USA).

2.5. Bioinformatic analysis

2.5.1. Read pre-processing and mapping

Three biological replicates were analyzed for *GMRGAL4/+* and *GMR > eIF6* larval eye imaginal discs and four biological replicates were analyzed for *GMRGAL4/+* and *GMR > eIF6* pupal retinae, for a total of 14 samples. Raw reads were checked for quality by FastQC software (version 0.11.2, S., A. FastQC: a quality control tool for high-throughput sequence data. 2010; Available from: http://www.bioinformatics.babrah

am.ac.uk/projects/fastqc), and filtered to remove low quality calls by Trimmomatic (version 0.32) (Bolger et al., 2014) using default parameters and specifying a minimum length of 50. Processed reads were then aligned to *Drosophila melanogaster* genome assembly GRCm38 (Ensembl version 79) with STAR software (version 2.4.1c) (Dobin et al., 2013).

2.5.2. Gene expression quantification and differential expression analysis

HTSeq-count algorithm (version 0.6.1, option -s = no, gene annotation release 79 from Ensembl) (Anders et al., 2015) was employed to produce gene counts for each sample. To estimate differential expression, the matrix of gene counts produced by HTSeq was analyzed by DESeq2 (version DESeq2_1.12.4) (Love et al., 2014). The differential expression analysis by the DeSeq2 algorithm was performed on the entire dataset composed by both larvae and pupae samples. The two following comparisons were analyzed: *GMR > eIF6 versus GMRGAL4/+* larval eye imaginal discs (6 samples overall) and *GMR > eIF6 versus GMRGAL4/+* pupal retinae (8 samples in total). Reads counts were normalized by calculating a size factor, as implemented in DESeq2. Independent filtering procedure was then applied, setting the threshold to the 62 percentile; 10886 genes were therefore tested for differential expression. Significantly modulated genes in *GMR > eIF6* genotype were selected by considering a false discovery rate lower than 5%. Regularized logarithmic (rlog) transformed values were used for heat map representation of gene expression profiles. Analyses were performed in R version 3.3.1 (2016-06-21, Computing, T.R.F.f.S. R: A Language and Environment for Statistical Computing. Available from: http://www.R-project.org/).

2.5.3. Functional analysis by topGO

The Gene Ontology enrichment analysis was performed using topGO R Bioconductor package (version topGO_2.24.0). The option *nodesize = 5* is used to prune the GO hierarchy from the terms which have less than 5 annotated genes and the *annFUN.db* function is used to extract the gene-to-GO mappings from the genome-wide annotation library *org.Dm.eg.db* for *D. melanogaster*. The statistical enrichment of GO was tested using Fisher's exact test. Both the "classic" and "elim" algorithms were used.

2.5.4. Gene set association analysis

Gene set association analysis for larvae and pupae samples was performed by GSAA software (version 2.0) (Xiong et al., 2014). Raw reads for 10886 genes identified by Entrez Gene ID were analyzed by GSAA-SeqSP, using gene set C5 (*Drosophila* version retrieved from http://www.go2msig.org/cgi-bin/prebuilt.cgi?taxid=7227) and specifying as permutation type 'gene set' and as gene set size filtering min 15 and max 800.

2.6. Western blotting and antibodies

Larval imaginal discs, pupal retinae and adult heads were dissected in cold Phosphate Buffer Saline (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 137 mM, KCl 2.7 mM, pH 7.4) (PBS) and then homogenized in lysis buffer (HEPES 20 mM, KCl 100 mM, Glycerol 5%, EDTA pH 8.0 10 mM, Triton-X 0.1%, DTT 1 mM) freshly supplemented with Protease Inhibitors (Sigma, St. Louis, MO, USA, #P8340). Protein concentration was determined by BCA analysis (Pierce, Rockford, IL, USA, #23227). Equal amounts of proteins were loaded and separated on a 10% SDS-PAGE, then transferred to a PVDF membrane. Membranes were blocked in 10% Bovine Serum Albumin (BSA) in PBS-Tween (0.01%) for 30 min at 37 °C. The following primary antibodies were used: rabbit anti-eIF6 (1:500, this study), rabbit anti-β-actin (1:4000, CST, Danvers, MA, USA, #4967; RRID: AB_330288), mouse anti-Puromycin (1:500, Merck Millipore, #MABE343; RRID: AB_2566826). To produce the anti-eIF6 antibody used in this study, a rabbit polyclonal antiserum against two epitopes on COOH-terminal peptide of eIF6 (NH2-CLSFVGMNTTATEI-COOH eIF6 203–215 aa; NH2-CATVTTKLRAALIEDMS-COOH eIF6 230–245 aa) was prepared by PrimmBiotech (Milan, Italy, Ab code: 201212-00003 GHA/12), purified in a CNBr-Sepharose column and tested for its specificity against a mix of synthetic peptides with ELISA test. The following secondary antibodies were used: donkey anti-mouse IgG HRP (1:5000, GE Healthcare, Little Chalfont, UK, Amersham #NA931; RRID: AB_772210) and donkey anti-rabbit IgG HRP (1:5000, GE Healthcare, Amersham #NA934; RRID: 772206).

2.7. SUnSET assay

Larval imaginal eye and wing discs were dissected in complete Schneider medium (Lonza, Basel, Switzerland) and treated *ex vivo* with puromycin (50 μg/mL) for 30 min at room temperature, then fixed in 3% paraformaldehyde (PFA) for 1 h at room temperature. Immunofluorescences were then performed as described below, using a mouse anti-puromycin (1:500, Merck Millipore, Billerica, MA, USA, #MABE343, RRID: AB_2566826) as a primary antibody. Discs were then examined by confocal microscope (Leica SP5, Leica, Wetzlar, Germany) and fluorescence intensity was measured with ImageJ software.

2.8. Cell count

GMRGAL4/+ and *GMR > eIF6* pupal retinae at 40h APF were dissected, fixed, and stained with anti-Armadillo to count cells, as previously described (Cordero et al., 2004). Cells contained within a hexagonal array (an imaginary hexagon that connects the centres of the surrounding six ommatidia) were counted; for different genotypes, the number of cells per hexagon was calculated by counting cells, compared with the corresponding control. Cells at the boundaries between neighbouring ommatidia count half. At least 3 hexagons (equivalent to 9 full ommatidia) were counted for each genotype, and phenotypes were analyzed. Standard Deviation (SD) and unpaired two-tailed Student t-test were used as statistical analysis.

2.9. Immunofluorescences, antibodies and TUNEL assay

Larval imaginal discs and pupal retinae were dissected in cold PBS and fixed in 3% paraformaldehyde (PFA) for 1 h at room temperature, then washed twice with PBS and blocked in PBTB (PBS, Triton 0.3%, 5% Normal Goat Serum and 2% Bovine Serum Albumin) for 3 h at room temperature. Primary antibodies were diluted in PBTB solution and incubated O/N at 4 °C. After three washes with PBS, tissues were incubated O/N at 4 °C with secondary antibodies and DAPI (1:1000, Molecular Probes, Eugene, OR, USA, #D3571) in PBS. After three washes with PBS, eye imaginal discs and retinae were mounted on slides with ProLong Gold (LifeTechnologies, Carlsbad, CA, USA, #P36930). The following primary antibodies were used: rabbit anti-eIF6 (1:50, this study), rat anti-ELAV (1:100, Developmental Study Hybridoma Bank DSHB, Iowa City, IA, USA, #7E8A10; RRID: AB_528218), mouse anti-CUT (1:100, DSHB, #2B10; RRID: AB_528186), mouse anti-Armadillo (1:100, DSHB, #N27A; RRID: AB_528089), mouse anti-Chaoptin (1:100, DSHB, #24B10; RRID: AB_528161), rabbit anti- Dcp-1 (1:50, CST, #9578; RRID: AB_2721060), mouse anti-Puromycin (1:500, Merck Millipore, #MABE343; RRID: AB_2566826). The following secondary antibodies were used: goat anti-rat, goat anti-mouse, goat anti-rabbit (1:500 Alexa Fluor® secondary antibodies, Molecular Probes; RRID: AB_142924; AB_143157; AB_141778). Dead cells were detected using the In Situ Cell Death Detection Kit TMR Red (Roche, Basel, Switzerland, #12156792910) as manufacturer protocol, with some optimizations. Briefly, retinae of the selected developmental stage were dissected in cold PBS and fixed with PFA 3% for 1 h at room temperature. After three washes in PBS, retinae were permeabilized with Sodium Citrate 0.1%-Triton-X 0.1% for 2 min at 4 °C and then incubated overnight at 37 °C with the enzyme mix. Retinae were then rinsed three times with PBS, incubated with DAPI to stain nuclei and mounted on slides. Discs and retinae were examined by confocal microscopy (Leica SP5) and analyzed with Volocity 6.3 software (PerkinElmer, Waltham, MA, USA).

2.10. Semithin sections

Semithin sections were prepared as described in (Montrasio et al., 2007). Adult eyes were fixed in 0.1 M Sodium Phosphate Buffer, 2% glutaraldehyde, on ice for 30 min, then incubated with 2% OsO_4 in 0.1 M Sodium Phosphate Buffer for 2 h on ice, dehydrated in ethanol (30%, 50%, 70%, 90%, and 100%) and twice in propylene oxide. Dehydrated eyes were then incubated O/N in 1:1 mix of propylene oxide and epoxy resin (Sigma, Durcupan™ ACM). Finally, eyes were embedded in pure epoxy resin and baked O/N at 70 °C. The embedded eyes were cut on a Leica UltraCut UC6 microtome using a glass knife and images were acquired with a 100X oil lens, Nikon Upright XP61 microscope (Nikon, Tokyo, Japan).

2.11. Ecdysone treatment

For ecdysone treatment, 20-HydroxyEcdysone (20HE) (Sigma, #H5142) was dissolved in 100% ethanol to a final concentration of 5 mg/mL; third instar larvae from different genotypes (*GMRGAL4/+* and *GMR > eIF6*) were collected and placed in individual vials on fresh standard cornmeal food supplemented with 240 μg/mL 20HE. Eye phenotype was analyzed in adult flies, and images were captured with a TOUPCAM™ Digital camera. Eye images were analyzed with ImageJ software.

2.12. In vitro Ribosome Interaction Assay (iRIA)

iRIA assay was performed as described in (Pesce et al., 2015). Briefly, 96-well plates were coated with a cellular extract diluted in 50 μL of PBS, 0.01% Tween-20, O/N at 4 °C in a humid chamber. The coating solution was removed and unspecific sites were blocked with 10% BSA, dissolved in PBS, 0.01% Tween-20 for 30 min at 37 °C. Plates were washed with 100 μL/well with PBS-Tween. 0.5 μg of recombinant biotinylated eIF6 were resuspended in a reaction mix: 2.5 mM $MgCl_2$, 2% DMSO and PBS-0.01% Tween, to reach 50 μL of final volume/well, added to the well and incubated with coated ribosomes for 1 h at room temperature. To remove unbound proteins, each well was washed 3 times with PBS, 0.01% Tween-20. HRP-conjugated streptavidin was diluted 1:7000 in PBS, 0.01% Tween-20 and incubated in the well, 30 min at room temperature, in a final volume of 50 μL. Excess of streptavidin was removed through three washes with PBS-Tween. OPD (o-phenylenediamine dihydrochloride) was used according to the manufacturer's protocol (Sigma-Aldrich) as a soluble substrate for the detection of streptavidin-peroxidase activity. The signal was detected after the incubation, plates were read at 450 nm on a multiwell plate reader (Microplate model 680, Bio-Rad, Hercules, CA, USA).

2.13. EIA assay

Ecdysone levels from eye imaginal discs and retinae were titred according to the 20HE Enzyme Immunoassay kit protocol (Bertin Pharma, Montigny le Bretonneux, France, #A05120.96). Standard curves were generated using 20HE provided by the kit. Absorbance was measured at 405 nm with Tecan Freedom EVO (Tecan, Männedorf, Switzerland).

2.14. Key resources table

Reagent or resource	Source	Identifier
Antibodies		
Rabbit anti-eIF6	This study	N/A
Rabbit anti-β-actin	Cell Signalling Technology	RRID:AB_330288
Mouse anti-Puromycin (clone 12D10)	Millipore	RRID:AB_2566826
Rat anti-Elav	DHSB	RRID:AB_528218
Mouse anti-Cut	DSHB	RRID:AB_528186
Mouse anti-Armadillo	DSHB	RRID:AB_528089
Mouse anti-Chaoptin	DSHB	RRID:AB_528161
Rabbit anti-Dcp-1	Cell Signalling Technology	RRID:AB_2721060
Sheep anti-mouse IgG-HRP	GE Healthcare	RRID:AB_772210
Donkey anti-rabbit IgG-HRP	GE Healthcare	RRID:AB_772206
Goat anti-mouse IgG, Alexa Fluor 488	Life Technologies	RRID:AB_142924
Goat anti-rabbit IgG, Alexa Fluor 568	Life Technologies	RRID:AB_143157
Goat anti-rat IgG, Alexa Fluor 647	Life Technologies	RRID:AB_141778
Chemicals, Peptides, and Recombinant Proteins		
Rapamycin	Sigma	Cat#R8781
Insulin	Sigma	Cat#I0516
Puromycin	ThermoFisher Scientific	Cat#A1113803
Protease Inhibitors	Sigma	Cat#P8340
20-HydroxyEcdysone	Sigma	Cat#H5142
DAPI	Molecular Probes	Cat#D3571
Durcupan™ ACM	Sigma	Cat#44610-1 EA
Critical Commercial Assays		
BCA Protein Assay Kit	Pierce	Cat#23227
In Situ Cell Death Detection Kit TMR Red	Roche	Cat#12156792910
SuperScript III First-Strand Synthesis SuperMix for qRT-PCR	Invitrogen	Cat#11752-050
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	ThermoFisher Scientific	Cat#34577
mirVana™ miRNA Isolation Kit	Life Technologies	Cat#AM1560
DNA-free™ DNA Removal Kit	Life Technologies	Cat#AM1906
Qubit® RNA Assay Kit	Life Technologies	Cat#Q32852
TaqMan® Universal PCR Master Mix	Life Technologies	Cat#4304437
GoTaq® qPCR Master Mix	Promega	Cat#A6001
TruSeq RNA Library Prep Kit v2	Illumina	Cat#RS-122-2001
SBS Kit v3	Illumina	Cat#FC-401-3001
Enzyme Immunoassay Kit	Bertin Pharma	Cat#A05120.96
Deposited Data		
Accession number ID	ArrayExpress	E-MTAB-5954
Experimental Models: Organisms/Strains		
D. melanogaster: *GMRGAL4/CTG*	A gift from Manolis Fanto (King's College, London)	N/A
D. melanogaster: *UAS-eIF6*	A gift from William J Brook (Alberta Children's Hospital, Calgary)	N/A
D. melanogaster: *y[1] w[*]; P{w [+mC] = tubP-GAL4}LL7/TM3, Sb [1] Ser[1]*	Bloomington Drosophila Stock center	BDSC: 5138
		BDSC: 26656

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Reagent or resource	Source	Identifier
D. melanogaster: *P{w [+mC] = spa-GAL4.J}1, w[*]*	Bloomington Drosophila Stock center	
D. melanogaster: *y[1] w[*]; P{w [+m*] = GAL4} 54C*	Bloomington Drosophila Stock center	BDSC: 27328
D. melanogaster: *w [1118]*	Bloomington Drosophila Stock center	BDSC: 3605
D. melanogaster: *w [1118] P{w*		*[+mW.hs] = GawB}Bx[MS1096]*
Bloomington Drosophila Stock center	BDSC: 8860	
Experimental Models: Cell Lines		
D. melanogaster: Schneider S2 cells	DGRC	RRID:CVCL_TZ72
Oligonucleotides		
Drosophila eIF6	Applied Biosystem	CAT#Dm01844498_g1
Drosophila RPL32	Applied Biosystem	CAT#Dm02151827_g1
Drosophila Shd	Metabion	F 5′-CGGATCGATATGCTAAGCTGT-3′, R 5′-CGACGCACTCYCYYGTCG-3′
Drosophila RPL32	Metabion	F 5′- TCTCGCTCTTGTCGTGTCTG-3′, R 5′- CCGATATCCTTCGCGTACTG -3′
Software and Algorithms		
R environment for statistical computing (version 3.3.1)	N/A	https://www.r-project.org/
FastQC (version 0.11.2)	Andrews, S. (2014)	http://www.bioinformatics.babraham.ac.uk/projects/fastqc
Trimmomatic (version 0.32)	Bolger, A. M. et al. (2014)	http://www.usadellab.org/cms/?page = trimmomatic
STAR software (version 2.4.1c)	Dobin, A. et al. (2013)	https://github.com/alexdobin/STAR
HTSeq-count (version 0.6.1)	Anders, S. et al. (2015)	https://pypi.org/project/HTSeq/
DESeq2 (version DESeq2_1.12.4)	Love, M.I. et al. (2014)	https://github.com/mikelove/DESeq2
topGO (version topGO_2.24.0)	Alexa, A. et al. (2016)	https://bioconductor.org/packages/release/bioc/html/topGO.html
GSAA (version 2.0)	Xiong, Q. et al. (2014)	http://gsaa.unc.edu/
Volocity (version 6.3)	Quorum Technologies	http://quorumtechnologies.com/index.php/2014-06-19-13-10-00/2014-06-19-13-14-30/image-analysis/2-uncategorised/110-volocity-downloads
Microsoft Excel	Microsoft	https://www.microsoft.com/
ImageJ	ImageJ	https://imagej.nih.gov/ij/
GraphPad 7	Prism	https://www.graphpad.com/scientific-software/prism/

3. Results

3.1. Increased eIF6 levels cause embryonic lethality and aberrant morphology

Regulation of eIF6 levels is stringent in normal conditions (Donadini et al., 2001), with evidence for eIF6 amplification (Gatza et al., 2014) and overexpression (Biffo et al., 1997; Miluzio et al., 2015; Harris et al., 2004; Martin et al., 2008; Rosso et al., 2004) in cancer. We used the *Drosophila melanogaster* model to establish whether an increased activity of eIF6 could drive specific developmental decisions.

First, we assessed the effects caused by the loss of the *eIF6 D. melanogaster* homologue. To this end, we used the *P* element allele $eIF6^{k13214}$ (Spradling et al., 1999), to induce mitotic clones homozygous for $eIF6^{k13214}$ in first instar larvae by heat shock-induced FLIP/*FLP*-mediated homologous recombination (Harrison and Perrimon, 1993). We did not observe clones of eIF6 mutant cells in all adult tissues, with the exception of small ones in the wing margin (S1A Fig). Similar results were obtained in a *minute* background that provides a growth advantage to mutant cells, or by targeted expression of FLP in the wing margin (S1A Fig). Together, these results confirm that eIF6 is required for cell viability in *Drosophila*, as previously observed in yeast (Sanvito et al., 1999) and mammals (Gandin et al., 2008), precluding significant studies on the effects of eIF6 inhibition.

Next, we assessed the effects of eIF6 high levels, by ubiquitous expression of *eIF6* under the *TubGAL4* driver. Ectopic expression resulted in late embryonic lethality (S1B Fig). To circumvent early lethality, we focused on a non-essential fly organ, the eye. Increased eIF6 expression during late larval eye disc development, driven by the *GMRGAL4* driver (*GMR > eIF6*), causes the formation of a reduced and rough adult eye (Fig. 1A). We developed a new antibody specific for *Drosophila* eIF6 and we estimated its protein levels (Materials and Methods section) was about doubled compared to control (Fig. 1B). The stereotypic structure of the wild-type eye was severely disrupted with flattened ommatidia and bristles arranged in random patterns as shown by SEM analysis (Fig. 1C). Semithin sections evidenced an aberrant morphology and arrangement of ommatidia (Fig. 1D). These data show that increasing eIF6 levels in the fly eye cause a disruption of eye development.

3.2. Increased eIF6 levels delay physiological apoptosis

To understand the origin of the defects observed in *GMR > eIF6* adult eyes, we analyzed eye development in larvae, starting from the third instar, the stage at which the GMR driver starts to be expressed. We found that third instar imaginal discs with high levels of eIF6 showed no differences in terms of morphology, cell identity or developmental delay when compared to control (S2A-B Fig). Then, we analyzed pupal development. In *GMR > eIF6* retinae at 40h after puparium formation (APF) both neuronal and cone cells were present in the correct numbers. However, ommatidial morphology was altered (S2C Fig). A fundamental event controlling ommatidial morphology is the developmentally-controlled wave of Programmed Cell Death (PCD), sweeping the tissue from 25h to 42h APF (Ready et al., 1976). Thus, we analyzed by immunostaining the expression of *Drosophila* apoptotic effector caspase Dcp-1, as a marker of PCD, at 40h APF. Control retinae showed a clear presence of apoptotic cells. Remarkably, apoptotic cells were reduced in *GMR > eIF6* retinae (Fig. 2A).

Dcp-1 positive cells, i.e. apoptotic cells, increased in *GMR > eIF6* retinae at 60h APF (Fig. 2B). In summary, quantification of the number of Dcp-1 positive cells at 40h APF and 60h APF in *GMR > eIF6* revealed up to 75% reduction in the number of apoptotic cells at 40h APF, and an increase at 60h APF retinae (Fig. 2C and D). A change in apoptosis dynamics was also visualized by TUNEL assay at 28h APF, the time at which PCD starts in control retinae. Here, we observed the reduction of apoptotic nuclei in the *GMR > eIF6* retinae, while *GMRGAL4/+* retinae showed several (S2D Fig). In conclusion, eIF6 overexpression either blocks the early apoptotic program or delays it. We stained for the *Drosophila* β-catenin homologue Armadillo (Fig. 3), which localizes to membranes of cells surrounding photoreceptors, providing an indication of ommatidial cell number. At 40h APF, control retinae presented the typical Armadillo staining. *GMR > eIF6* retinae showed the presence of extra-numerary cells around the ommatidial core (Fig. 3A), indicating that interommatidial cells (IOCs) were not removed by PCD. By counting the number of cells in each ommatidium, we determined that *GMR > eIF6* retinae possess more than 13 cells, corresponding to approximately 30% more than that of a wild-type ommatidium (S3A Fig). Later in development, at 60h and at 72h APF, in *GMR > eIF6* retinae Armadillo was no longer detectable, while in wild-type retinae the pattern of Armadillo was maintained (Fig. 3B and S3B Fig). These data indicate that delayed PCD in *GMR > eIF6* inappropriately removes most IOCs. We suggest that the first effect of eIF6 high levels is an early block

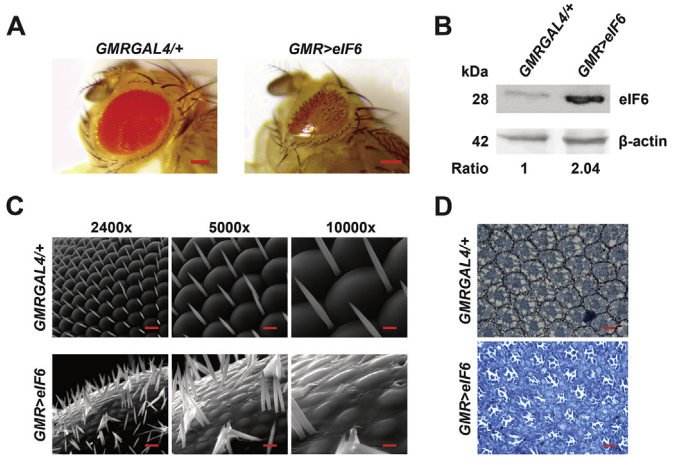

Fig. 1. Increased eIF6 levels in the developing eye result in a rough eye phenotype (A) Representative stereomicroscope images of *GMRGAL4/+* and *GMR > eIF6* eyes, showing a rough eye phenotype. Scale bar 300 μm. **(B)** Western blot showing the levels of eIF6 expression in *GMRGAL4/+* and *GMR > eIF6* adult eyes. Representative western blots from three independent experiments are shown. Molecular weight markers (kDa) are shown to the left of each panel. The ratio was calculated with ImageJ software. The value corresponds to the intensity ratio between eIF6 and β-actin bands for each genotype. **(C)** Representative SEM images of *GMRGAL4/+* and *GMR > eIF6* adult eyes. eIF6 overexpressing eyes have an aberrant morphology, showing flattened ommatidia and randomly arranged bristles. Scale bar, respectively for 2400X, 5000X and 10000X magnifications are 10 μm, 5 μm and 2.5 μm **(D)** Representative tangential sections of *GMRGAL4/+* and *GMR > eIF6* adult eyes indicating that photoreceptors are still present in *GMR > eIF6* eyes, even if their arrangement is lost. Scale bar 10 μm.

of apoptosis that leads in turn to an aberrant developmental program.

3.3. Increased eIF6 expression in cone cells is sufficient to delay apoptosis

Cone cells and IOCs are known to be the main actors during physiological PCD (Rusconi et al., 2000). We overexpressed eIF6 under the control of the cone cell-specific driver, *spaGAL4*. We observed a milder phenotype compared to *GMR > eIF6* adult eyes (Fig. 4A–B and S4A Fig). Importantly, eIF6 overexpression in cone cells (S4B Fig) caused reduced Dcp-1 staining in 40h APF retinae (Fig. 4C), and evident apoptosis at 60h APF (Fig. 4D), in line with what we observed in *GMR > eIF6* retinae. Thus, the expression of eIF6 in cone cells is sufficient to alter PCD and cause defects in eye development.

3.4. eIF6 expression reshapes the transcriptome, increases ribosome activity and represses ecdysone signalling

Next, we asked whether eIF6 was associated with a transcriptional rewiring that could account for the observed phenotypic effects. To this end, we performed a comprehensive gene expression analysis of *GMRGAL4/+* and *GMR > eIF6* genotypes at two distinct stages of eye development, larval eye imaginal discs and pupal retinae, by RNA-Seq (Fig. 5). In *GMR > eIF6* samples at both developmental stages, we observed an upregulation of genes involved in ribosome biogenesis (Fig. 5A, S1 File). GSAA analysis revealed also an increase in mRNAs of genes involved in rRNA processing (Fig. 5C). Overall these data suggest that eIF6 is able to increase ribosomal gene expression.

Consistent with our phenotypic analysis of the eye, *GMR > eIF6* retinae displayed also variations in genes involved in eye development and in PCD (Fig. 5A, D and S1 File). Notably, mRNAs encoding specialized eye enzymes, such as those of pigment biosynthetic pathways, were downregulated in *GMR > eIF6* samples (S1 File), preceding the altered adult eye morphology.

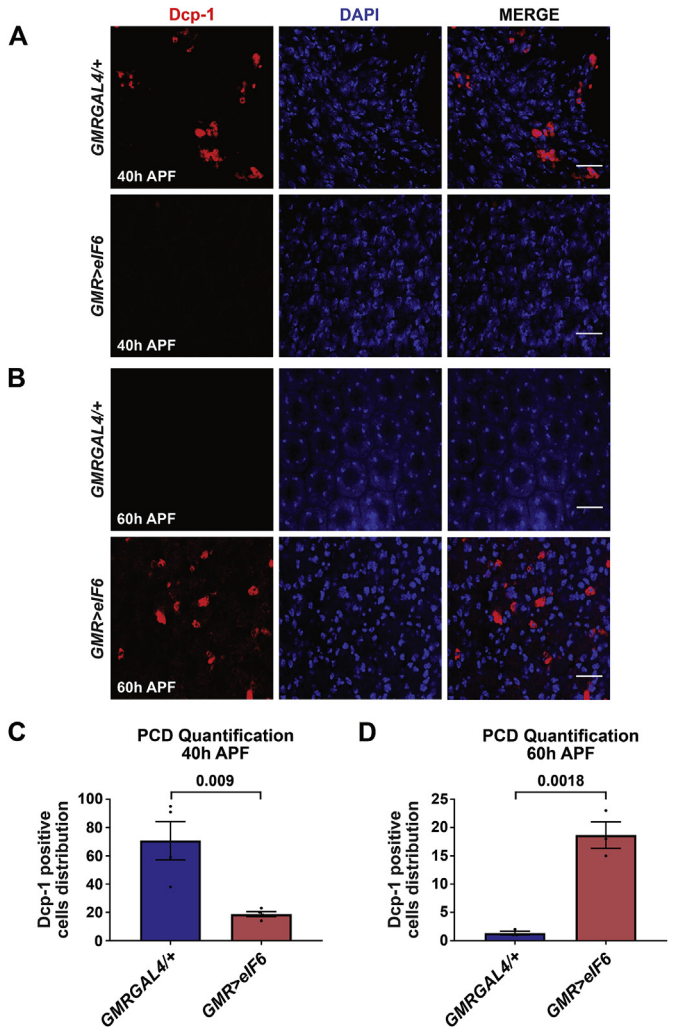

Fig. 2. The apoptotic wave is delayed when eIF6 levels are increased. (A) Mid-pupal stage retinae (40h APF) stained for the *Drosophila* caspase Dcp-1. *GMRGAL4/+* retinae show Dcp-1 positive cells, indicating that PCD is ongoing at this developmental stage. On the contrary, *GMR > eIF6* retinae do not show Dcp-1 positive cells, indicating a block in PCD. Scale bar 10 μm. **(B)** Late-pupal stage (60h APF) retinae stained for the *Drosophila* caspase Dcp-1. *GMRGAL4/+* retinae show a reduction of Dcp-1 positive cells, as expected (PCD already finished at this developmental stage). On the contrary, *GMR > eIF6* retinae, show Dcp-1 positive cells, indicating a delay in PCD associated with more eIF6 levels. Scale bar 10 μm. **(C–D)** Barplot showing the Dcp-1 positive cells counts average from four different areas (n = 4) at 40h APF **(C)** and 60h APF **(D)** retinae with error bars indicating the SEM. P-values were calculated using an unpaired two-tailed Student t-test. Dcp-1 positive cells count indicates an overall delay and increases in PCD when eIF6 is increased during eye development.

Finally, coordinated changes induced by eIF6 in eye imaginal discs surprisingly clustered into the ecdysone pathway, with a striking downregulation of many enzymes involved in 20-HydroxyEcdysone (20HE) biosynthesis (Fig. 5A and B). For instance, expression of *phm*, *sad* and *nvd* (S5 Fig) was virtually absent in *GMR > eIF6* eye imaginal disc, while early (*rbp*) and late (*ptp52f*) responsive genes belonging to the hormone signalling cascade were downregulated (S1 File). In conclusion, our gene expression analysis of *GMR > eIF6* eye samples identifies a rewiring of transcription that is consistent with altered PCD, accompanied by upregulation of ribosomal genes and downregulation of the ecdysone biosynthetic pathway.

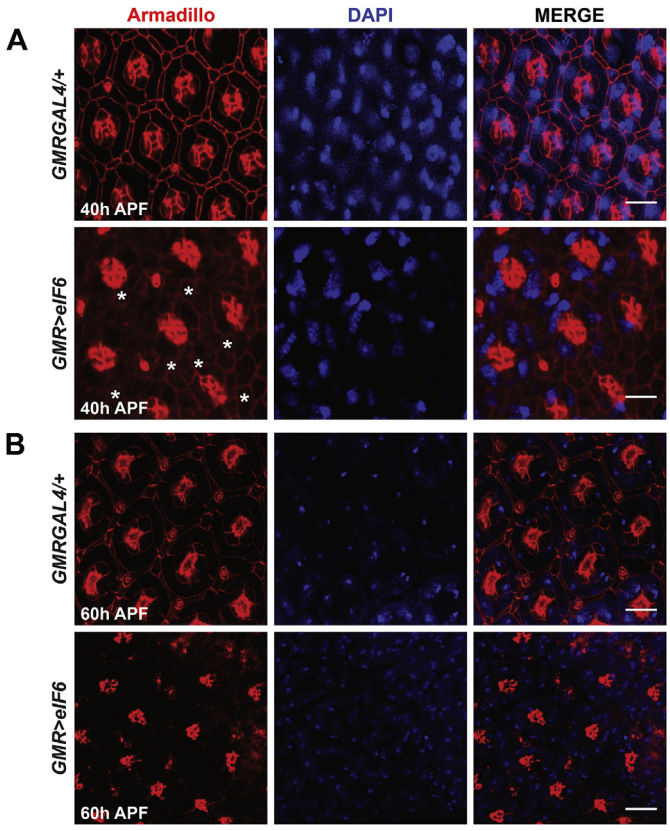

Fig. 3. Cell number is altered during the pupal stage in *GMR > eIF6* retinae. (A) Mid-pupal stage (40h APF) retinae stained for Armadillo, the *Drosophila* β-catenin homologue, showing that when eIF6 is increased there are extra-numerary cells (indicated as *) around each ommatidium. **(B)** Late-pupal stage (60h APF) retinae stained for Armadillo, showing the loss of all cells around ommatidia upon eIF6 overexpression. **(A–B)** Scale bar 10 μm.

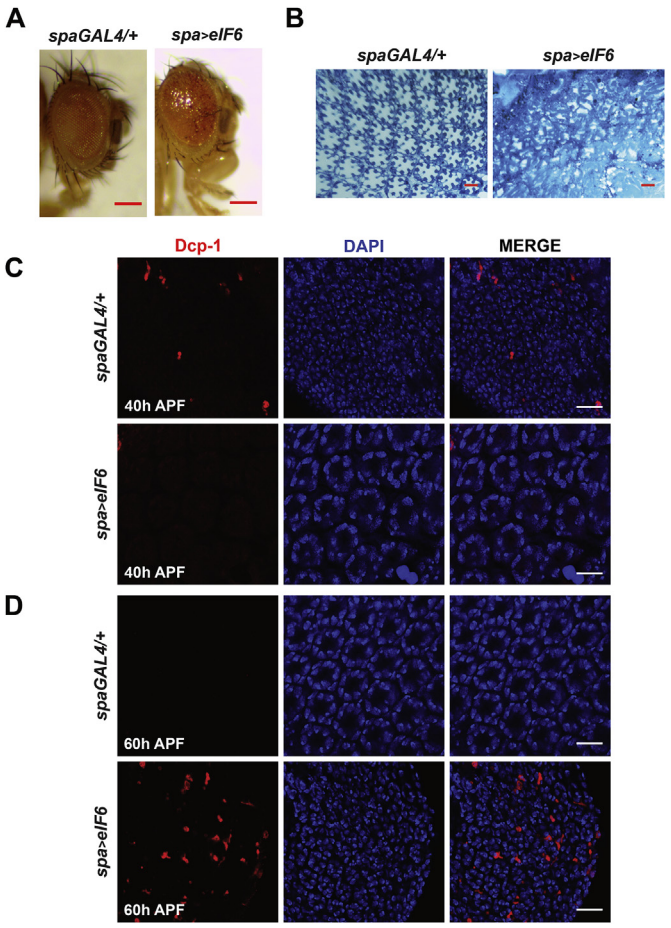

Fig. 4. A specific increase of eIF6 in cone cells results in a rough eye phenotype. (A–B) Overexpression of eIF6 in cone cells results in rough eye phenotype. **(A)** Representative stereomicroscope images of *spaGAL4/+* and *spa > eIF6* eyes showing a rough eye phenotype. Scale bar 300 μm **(B)** Representative tangential semithin sections of *spaGAL4/+* and *spa > eIF6* adult eyes showing disruption of the structure upon eIF6 overexpression in cone cells. Scale bar 10 μm. **(C)** Mid-pupal stage (40h APF) retinae of *spaGAL4/+* and *spa > eIF6* genotypes stained for Dcp-1 confirm the block in apoptosis already demonstrated in *GMR > eIF6* retinae. **(D)** Late-pupal stage (60h APF) retinae of *spaGAL4/+* and *spa > eIF6* genotypes stained for Dcp-1 confirming the delayed and increased apoptosis already observed in *GMR > eIF6* retinae. **(C–D)** Scale bar 10 μm.

3.5. Increased eIF6 levels result in elevated translation

eIF6 binds free 60S *in vitro* and *in vivo* affecting translation (Brina et al., 2015a). To assess whether increased transcription of genes related to ribosome biogenesis and rRNA processing observed in gene expression analysis experiments was accompanied by an effect on the translational machinery, we investigated changes in levels of free 60S subunits upon eIF6 overexpression. To this end, we performed the *in vitro* Ribosome Interaction Assay (iRIA) (Pesce et al., 2015), able to measure quantitative binding of proteins to ribosomes. We found that the expression of eIF6 in *GMR > eIF6* larval eye discs led to a 25% reduction in free 60S sites when compared to control (Fig. 6A). Next, we used a modified SUnSET assay (Schmidt et al., 2009), as a proxy of the translational rate. We measured translation in eye imaginal discs treated *ex vivo* with puromycin, which incorporates in nascent protein chains by ribosomes. Remarkably, *GMR > eIF6* eye discs incorporated almost twice the amount of puromycin, relative to control (Fig. 6B and C). Taken together, high levels of eIF6 increase the free 60S pool *in vivo*, and increase puromycin incorporation, i.e. translation.

We next determined whether the increase of translation, altered morphology and apoptosis correlate with heightened eIF6 levels in other organs. Thus, we overexpressed eIF6 in the wing imaginal disc, using the *bxMS1096GAL4* driver (*MS > eIF6*) (S6A Fig). Such manipulation led to complete disruption of the adult wing structure (Fig. 6D). Moreover, we performed the SUnSET assay on wing imaginal discs, and, as in eye discs, we observed a two-fold increase in puromycin incorporation in *MS > eIF6* wing discs' respect to control (Fig. 6E and S6B Fig). Finally, eIF6 overexpression in wing discs led to an increase of apoptotic cells in the dorsal portion of the disc (S6C Fig), as observed in 60h APF *GMR > eIF6* retinae. In conclusion, high levels of eIF6 lead not only to augmented expression of ribosomal genes, but also to augmented translational activity.

3.6. 20HE administration rescues adult eye defects induced by increased eIF6 levels

Transcriptome analysis revealed a coordinated shut-down of the 20HE biosynthetic pathway raising the question whether 20HE administration could at least partly rescue the defects driven by eIF6 increased levels, and a rough eye phenotype characterized by aberrant PCD. To determine the hierarchy of events that eIF6 overexpression causes, we administrated 20HE by feeding *GMR > eIF6* third instar larvae and we evaluated the effect on eye development. Remarkably, *GMR > eIF6* larvae fed with 20HE showed eyes that were 20% larger than untreated controls (Fig. 7A and B). We also assessed the levels of apoptosis at 40h APF. Notably, immunofluorescence staining for Dcp-1 showed the presence of apoptotic cells in 40h APF *GMR > eIF6* retinae treated with 20HE, while *GMR > eIF6* untreated retinae did not show any Dcp-1 positive cells (Fig. 7C). Taken together, these data suggest that the

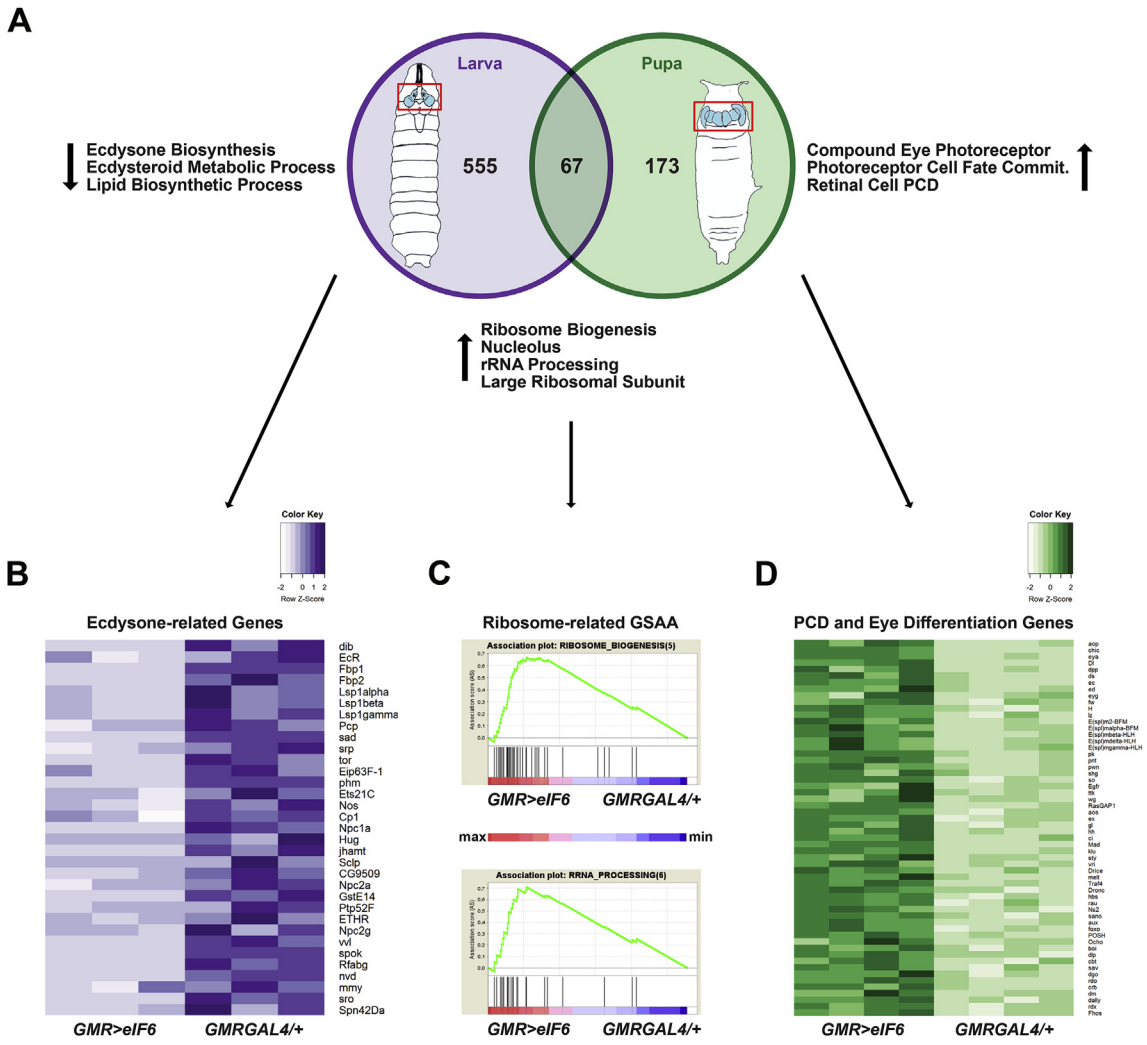

Fig. 5. eIF6 induces a reshaping of transcription, resulting in rRNA processing alteration and in a gene signature specific for the eye (A) Venn Diagram indicating genes differentially expressed in *GMR > eIF6* larval eye imaginal discs and *GMR > eIF6* retinae with respect to control (*GMRGAL4/+*). **(B)** The Ecdysone Biosynthetic Pathway is shut-down when eIF6 is upregulated. Heat Map representing absolute gene expression levels in *GMR > eIF6* and *GMRGAL4/+* eye imaginal disc samples for the subset of gene sets involved in Ecdysone Biosynthesis by Gene Ontology analysis. **(C)** Gene Set Association Analysis (GSAA) indicates a significant upregulation of the ribosomal machinery. Representative Enrichment Plots indicating a striking upregulation of genes involved in rRNA Processing (NAS: 2.24; FDR: 6,84E10-4) and Ribosome Biogenesis (NAS: 2.10; FDR: 0,013) in both *GMR > eIF6* eye imaginal discs and *GMR > eIF6* retinae with respect to their control (*GMRGAL4/+*). **(D)** mRNAs involved in Programmed Cell Death and in Eye Differentiation are upregulated in *GMR > eIF6* retinae. Heat Map representing absolute gene expression levels in *GMR > eIF6* and *GMRGAL4/+* retinae samples for the subset of gene sets involved in Programmed Cell Death and Eye Differentiation by Gene Ontology Analysis.

apoptotic defect and eye roughness caused by eIF6 overexpression are at least partly due to the inactivation of ecdysone signalling, that precedes deregulation of PCD.

3.7. *eIF6 and translation antagonize ecdysone biosynthesis during development*

Our findings indicate that increased eIF6 levels cause downregulation of mRNAs belonging to the ecdysone biosynthetic pathway, and the relative absence of its final product, the 20HE. To understand the physiological relevance of this phenomenon, we measured mRNAs levels of *eIF6* and *shd* at different stages of development (Fig. 7). *Shd* encodes for the last enzyme of the 20HE biosynthesis and it is specifically expressed in ecdysone target tissues (Petryk et al., 2003). Real-Time PCR evidenced the downregulation of *shd* in eye imaginal disc overexpressing eIF6 (Fig. 7D). We then investigated the levels of *eIF6* and *shd* during development in wild-type tissues (Fig. 7E and F). Interestingly, we found that eIF6 levels are regulated during development, and that *shd* levels drop when *eIF6* levels are high, both in embryos and first instar larvae (Fig. 7E) or first and third instar larvae (Fig. 7F). Importantly, 20HE levels drop at 40h APF retinae upon eIF6 overexpression (Fig. 7G). Taken together, data suggest that physiological eIF6 levels are inversely correlated with 20HE production.

Taken that high levels of eIF6 lead to an increase in general translation, we decided to study the relationship between the translational rate and ecdysone production in a physiological context. We assessed levels of *shd* and *EcR* (as an index of the feed-forward loop induced by 20HE itself (Niwa and Niwa, 2016)) mRNA levels in S2 cells after treatment with rapamycin or insulin to inhibit or stimulate translation respectively (Fig. 7H–J). After insulin treatment, we observed the downregulation of *shd* and *EcR* mRNA levels (Fig. 7H). Conversely, after rapamycin treatment, we found an upregulation of the two analyzed genes (Fig. 7J). These data support a physiological model in which translation is a negative regulator of ecdysone metabolism.

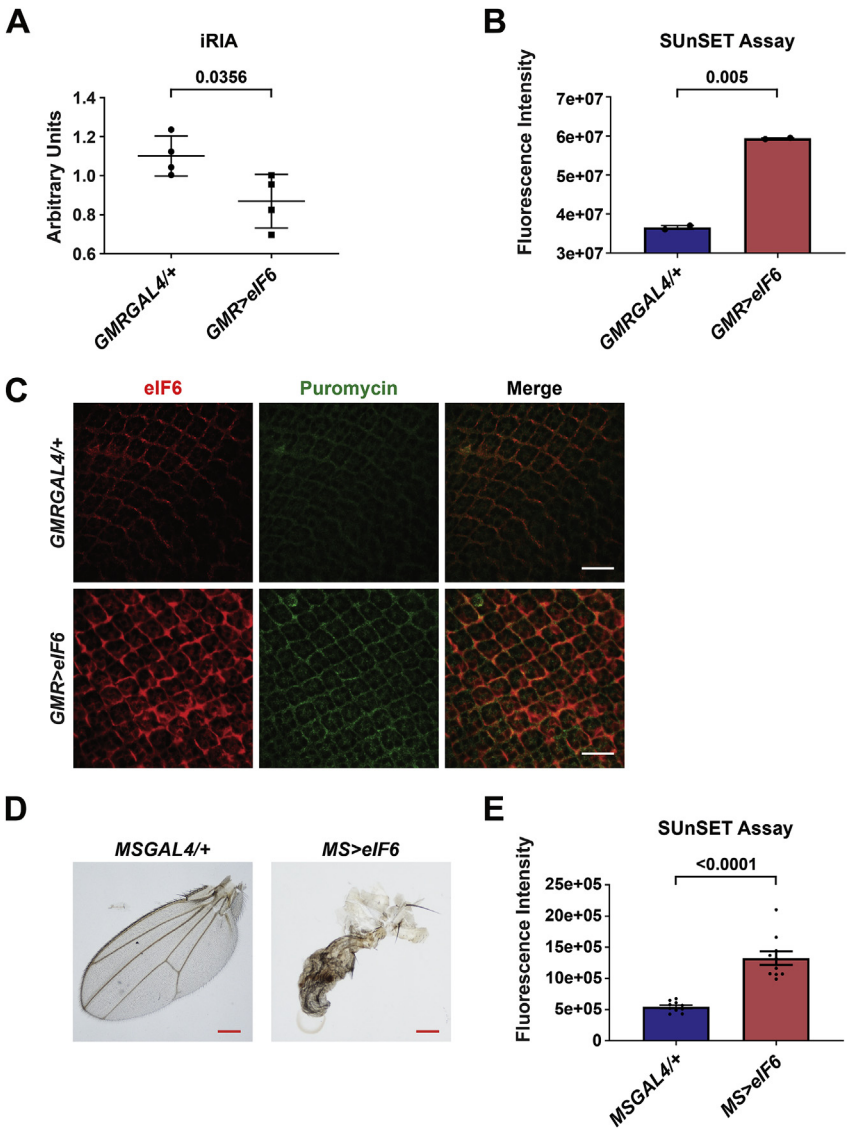

Fig. 6. Increased eIF6 levels in the developing eye result in reduced free 60S and increased translation. (A) iRIA assays showing that *eIF6* increased dosage reduce the number of free 60S subunits. Values represent the mean ± SEM from two replicates. Assays were repeated three times. Student's t-test was used to calculate p-values. **(B)** *In vitro* SUnSET assays showing that *eIF6* increased gene is associated with increased puromycin incorporation. Barplots represent the mean ± SEM from three replicates. Assays were repeated three times. Student's t-test was used to calculate p-values. Quantification of SUnSET assay was performed with ImageJ software. **(C)** Representative SUnSET assay performed using immunofluorescence experiments, indicating a two-fold increase in general translation when eIF6 levels are increased in eye imaginal discs. Scale bar 10 μm. **(D)** Adult wings *MS* > *eIF6* have a completely aberrant phenotype. Scale bar 200 μm. **(E)** *In vitro* SUnSET assays showing that *eIF6* increased gene is associated with 2-fold puromycin incorporation in wing discs. Barplots represent the mean ± SEM from three replicates. Assays were repeated three times. Student's t-test was used to calculate p-values. Quantification of SUnSET assay was performed with ImageJ software.

4. Discussion

The eukaryotic Initiation Factor 6 (*eIF6)* is an evolutionarily conserved gene encoding for a protein necessary for ribosome biogenesis and translation initiation (Gandin et al., 2008; Ceci et al., 2003). However, in mammals, eIF6 expression differs among tissues, with high levels in embryos and in cycling cells and almost undetectable levels in post-mitotic cells (Donadini et al., 2001). Developmental studies in mice demonstrated that null alleles for this initiation factor are incompatible with life (Gandin et al., 2008), whereas eIF6 haploinsufficiency is linked to an impairment in G1/S cell cycle progression (Gandin et al., 2008). In unicellular models, eIF6 mutations rescue the quasi-lethal phenotype due to loss of ribosome biogenesis factors such as SBDS (Menne et al., 2007). Taken together, these data highlight how eIF6 expression, despite its ubiquitous function, is strictly regulated. Indeed, we found that doubling levels of eIF6 during development disrupts eye morphology, increases translation and changes gene expression. Overall, our data demonstrate that eIF6 is a translation factor able to drive a complex transcriptional reshaping.

Mechanistically, eIF6 binds to the 60S in the intersubunit space, interacting with rpL23 and to the sarcin-loop (SRL) of rpL24 (Klinge et al., 2011), thus generating a steric hindrance that prevents the formation of an intersubunit bridge (Weis et al., 2015). *In vitro*, eIF6 can repress translation (Russell and Spremulli, 1980). In mice, however, high levels of eIF6 are required for both tumor progression (Miluzio et al., 2011), and insulin-controlled translation (Brina et al., 2015a; Gandin et al., 2008). In *Drosophila*, we found that the overexpression of eIF6 leads to a reduction of the free 60S pool in eye imaginal discs, consistent with eIF6 biochemical activity. Such reduction could imply lower general translation, due to less availability of 60S subunits, as in the case of *Sbds* mutants (Calamita et al., 2017). Conversely, 60S could be already engaged with 40S into active translating 80S, thus heightening general translation. We favour the latter hypothesis because, by a puromycin incorporation assay, we observe a two-fold increase in general translation, both in the developing eye and the wing. Intriguingly, the transcriptome signature associated with high levels of eIF6 revealed also an increase in mRNAs encoding for rRNA processing factors, suggesting that ribosome biogenesis is positively affected by eIF6. In conclusion, we surmise that *in vivo* eIF6 may act as a powerful stimulator of ribosome synthesis and translation.

The effects associated with increased translation driven by eIF6 are at least two, a change in the ecdysone pathway and a delay in apoptosis. We found a strong reduction of ecdysone biosynthesis pathway in the eye imaginal disc driven by eIF6. Importantly, 40h APF retinae evidence a reduction in hormone levels and 20HE administration leads to a partial rescue of the developmental defects driven by eIF6 increased activity.

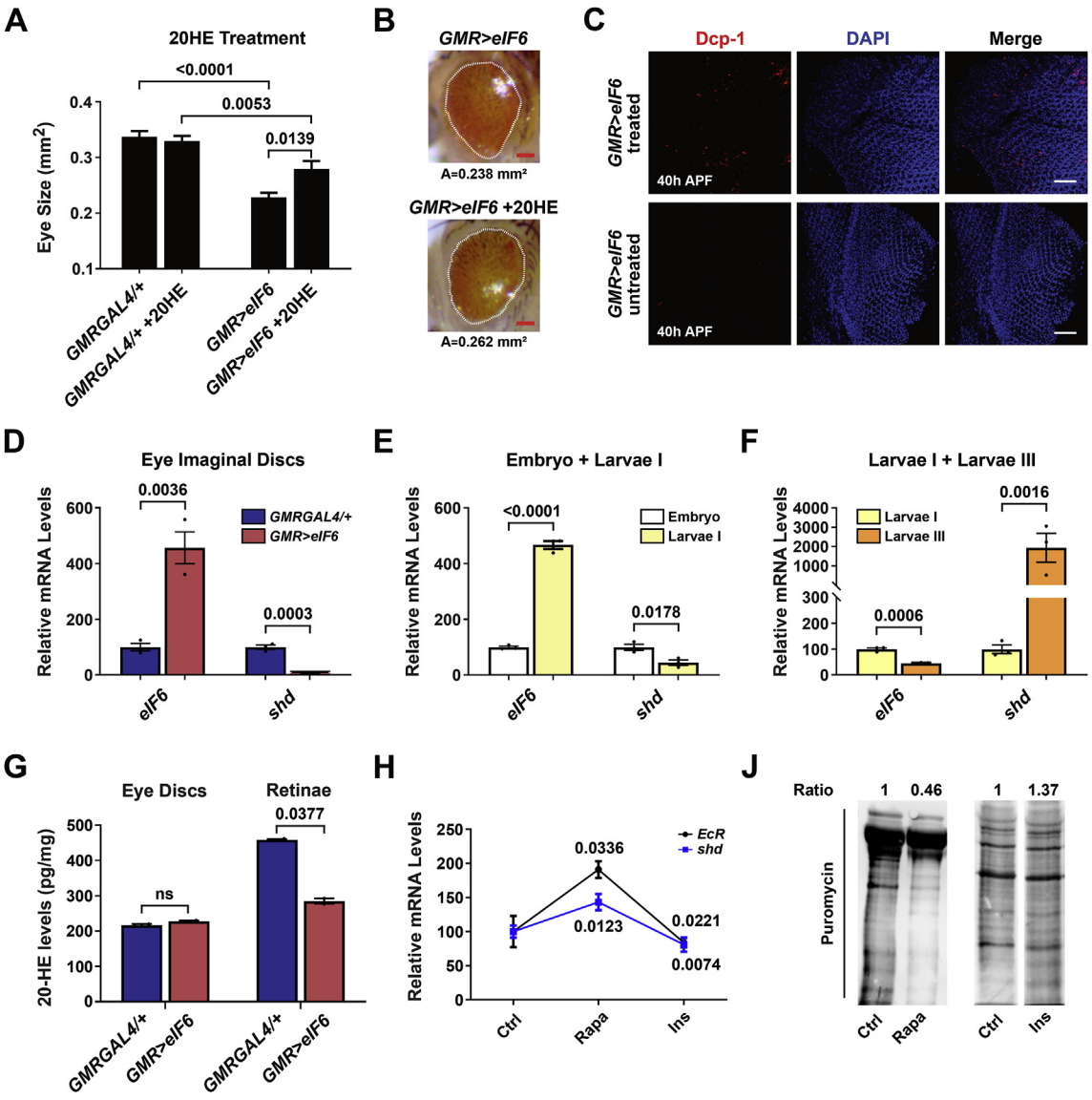

Fig. 7. 20HE treatment rescues the rough eye phenotype due to high levels of eIF6, unveiling the role of translation in ecdysone biosynthesis regulation. **(A–C)** 20HE treatment partially rescue the rough eye phenotype and the delay in apoptosis in 40h APF retinae **(A)** The barplot represents the average of n > 8 independently collected samples with error bars indicating the SEM. P-values were calculated using an unpaired two-tailed Student t-test. The graph shows the *GMR > eIF6* adult fly eye size with or without treatment with 20HE. As indicated in the barplot, the fly eye size is partially rescued when the hormone is added to the fly food. **(B)** Representative stereomicroscope images of *GMR > eIF6* eyes treated (upper panel) or untreated (lower panel) with 20HE, showing a partial rescue of the eye size when 20HE has been added. Scale bar 100 μm **(C)** Immunofluorescence images showing that 20HE treatment (240 μg/mL in standard fly food) rescues the apoptotic delay observed in *GMR > eIF6* 40h APF retinae. Scale bar 50 μm **(D–F)** Real-time PCR analyses of the indicated genes showing an inverse correlation between *eIF6* and *shd* mRNA levels. The RNA level of each gene was calculated relative to *RpL32* expression as a reference gene. The barplot represents the average of at least three independent biological replicates with error bars indicating the SEM. p-values were calculated using an unpaired two-tailed Student t-test. **(D)** Real-time PCR analyses of the indicated genes in *GMRGAL4/+* and *GMR > eIF6* eye imaginal discs. Upon eIF6 overexpression, *GMR > eIF6* eye imaginal discs have less abundance of *shd* mRNA levels compared to *GMRGAL4/+* eye imaginal discs. **(E–F)** During development, *eIF6* and *shd* mRNA levels show an inverse correlation by comparing embryos with first instar larval RNA extracts **(E)** or by comparing first and third instar larval RNA extracts **(F)**. **(G)** Ecdysone titers in *GMR > eIF6 and GMRGAL4/+* eye imaginal discs and 40h APF retinae. 20HE levels decrease in 40h APF *GMR > eIF6* retinae respect to control retinae. **(H–J)** The ecdysone biosynthetic pathway genes *shd* and *EcR* are modulated upon translation modulation in S2 cells. **(H)** Real time analysis evidences that upon inhibition of translation with rapamycin treatment (1 μM, 2 h) the level of *shd* and *EcR* mRNA levels increase, contrary to the drop observed upon translation stimulation with insulin (1 μM, 12 h). The RNA level of each gene was calculated relative to *RpL32* expression as a reference gene. The barplot represents the average of at least three independent biological replicates with error bars indicating the SEM. p-values were calculated using an upaired two-tailed Student t-test. **(J).** Representative Western blot showing the decreased or increased rate of protein synthesis upon rapamycin or insulin treatment respectively with SUnSET method (Schmidt et al., 2009).

Thus, our data suggest that eIF6 is upstream of ecdysone regulation. It has been recently suggested how translation regulation and hormonal signalling are tightly interconnected in *Drosophila* (Rode et al., 2018) and, more generally, that translation is a controller of metabolism (Biffo et al., 2017; Calamita et al., 2018). Our experiments unveil an inverse correlation between translational capability and ecdysone production. Concerning apoptosis we showed that eIF6 expression leads to an early block in Programmed Cell Death, as previously demonstrated by others in *X. laevis* (De Marco et al., 2010). The developmental defects driven by increased eIF6 levels are consistent with two scenarios: excess eIF6 could delay developmental PCD. Alternatively, PCD could be repressed at the correct developmental time and apoptotic elimination of defective cells

overexpressing eIF6 could be triggered later independently of developmental signals. The fact that overexpression of eIF6 in wing discs, which are not subjected to a developmental wave of apoptosis, leads to cell death, supports the latter hypothesis.

The developmental changes due to eIF6-driven translation are dramatic and include lethality, as well as disruption of development. In the past, similar effects were observed by the expression of another rate-limiting factor in translational initiation, eIF4E (Hernandez et al., 2005). It is unknown whether the developmental defects driven by eIF4E overexpression also included the arrest of ecdysone biosynthetic pathway, or an apoptotic block. However, in mammalian models, eIF4E and eIF6 share the common property of being rate-limiting for tumor growth and translation in several contexts (Carter et al., 2016; De Benedetti and Harris, 1999; Lazaris-Karatzas et al., 1990; Martinez-Saez et al., 2016; Oblinger et al., 2016; Robichaud and Sonenberg, 2017; Ruggero, 2013).

The signalling to eIF6 is different from signalling to eIF4E (Loreni et al., 2014), but the effects of inhibition of eIF4F complex by rapamycin are similar to eIF6 inhibition (Gandin et al., 2008; Beretta et al., 1996). This result may reflect the fact that both eIF6 and eIF4F converge on similar metabolic pathways like lipid synthesis (Brina et al., 2015b; Biffo et al., 2017) In summary, our study demonstrates that overexpression of eIF6 in developing organs is sufficient to induce an increase in ribosome biogenesis and translation that correlates with complex transcriptional and metabolic changes leading to hormonal and apoptotic defects. It will be interesting to further dissect the relationship between epigenetic, metabolic, and transcriptional changes associated with heightened eIF6 levels. Furthermore, our model may also be useful for *in vivo* screenings of compounds that suppress the effect of eIF6 overexpression.

Accession number

The RNA-Seq data are available at www.ebi.ac.uk/arrayexpress with accession number ID: E-MTAB-5954.

Acknowledgements

We thank William Brook (Alberta Children's Hospital, Calgary) for *UASeIF6* stocks and Manolis Fanto (King's College, London) for stocks and suggestions. We thank Valeria Berno for imaging help and Vera Giulia Volpi for semithin sections preparation. We thank Alan Warren (MRC, Cambridge) for useful suggestions. This work was supported by ERC TRANSLATE 338999, AIRC R0065-2 and Fondazione Cariplo to SB. PC is supported by Fondazione Umberto Veronesi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.05.013.

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