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Intrinsic oncogene silencing limits RAS-driven leukemogenesis through translational control

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Abstract

Oncogene-driven hematopoietic transformation is a dynamic process with poorly understood regulatory mechanisms. Using the *mpx/eGFP-HRAS* zebrafish model of myeloid leukemia, we investigated neutrophil responses during “oncogene silencing,” a phenomenon characterized by the transient expansion and subsequent disappearance of eGFP-HRAS-expressing neutrophils. To elucidate the molecular basis of this process, we performed single-cell RNA sequencing (scRNA-seq) of whole larvae and subsequently analyzed neutrophil populations at the onset of silencing. This analysis revealed transcriptional heterogeneity among neutrophils, identifying populations with varying oncogene expression and suggesting that some cells actively suppress oncogene activity. Among these, *Ctnnb1* and *Eif4ebp1* emerged as candidate regulators of neutrophil fate. Functional studies demonstrated that pharmacological inhibition of Wnt/ β -catenin signaling did not alter neutrophil dynamics, whereas CRISPR/Cas9-mediated disruption of *Eif4ebp1* impaired oncogene silencing, leading to persistent neutrophilia. Conversely, targeting its binding partner *Eif4e1c* reduced neutrophil numbers in both oncogenic and wild-type (WT) genetic contexts, while overexpression of WT versus non-phosphorylatable *Eif4e1c* variants produced opposing effects on neutrophil abundance. Since Mtor-mediated phosphorylation inactivates *Eif4ebp1*, we reasoned that Mtor inhibition could reinforce its translational repression activity. Indeed, treatment with rapamycin, an FDA/EMA-approved Mtor inhibitor, nearly abolished the expansion of transformed neutrophils, strongly potentiating oncogene silencing. Together, these findings identify the Mtor–*Eif4ebp1*–*Eif4e1c* translational axis as a central regulator of oncogene silencing and establish translational control as a therapeutically targetable mechanism capable of limiting RAS-driven myeloid transformation.

Keywords: Myeloid leukemia; oncogene silencing; single-cell RNA sequencing; zebrafish model; translational control; MTOR-EIF4E–EIF4EBP1 axis; RAS-driven leukemogenesis.

INTRODUCTION

The RAS GTPase superfamily comprises low-molecular-weight GTP-binding proteins that mediate the transduction of extracellular signals into diverse cellular processes. The main members of this superfamily include HRAS, NRAS, and KRAS (Castellano & Santos, 2011). In response to external stimuli, such as growth factors, cytokines, hormones, and environmental stressors, cell membrane receptors, known as receptor tyrosine kinases (RTKs), are activated upon ligand binding (Akinleye et al., 2013). This leads to RAS activation, which occurs through a reversible nucleotide exchange mechanism. In its active state (“on state”), RAS is associated with GTP, while in its inactive form (“off state”), it is bound to GDP. The conformational change from the inactive to the active state of RAS molecules significantly increases their affinity for their downstream effectors (Castellano & Santos, 2011; Herrmann, 2003). Among them, RAF MEK/ERK and PI3K PI3K/AKT/mTOR (PAM) are the most studied and well-characterized (Herrmann, 2003), each initiating distinct downstream signaling pathways.

The eIF4F complex controls cap-dependent mRNA translation in eukaryotic cells, as it is required to activate mRNAs and directly recruit the small subunit of the ribosome to them (Kim et al., 2024; Malka-Mahieu et al., 2017). It is composed of three proteins: the scaffolding factor eIF4G, the DEAD-box RNA helicase eIF4A, and the cap binding protein eIF4E. The latter constitutes the main component of the complex, as it is the responsible for binding to the 5’7-methyl guanosine (m⁷G) cap, located at the 5’ end of mRNAs (Malka-Mahieu et al., 2017; Raught & Gingras, 1999). eIF4E enhances the translation of several proteins involved in angiogenesis, cell cycle regulation, and apoptosis. Its activity is mainly regulated by the RAF/MEK/ERK pathway, through MAPK-interacting kinases 1/2 (MNK1/2), and by the PAM pathway, through mTOR/4E-BPs (Hsieh & Ruggero, 2010; Muta et al., 2011; Shi et al., 2015). On the one hand, ERK1/2 phosphorylate and, therefore, activate MNK1/2, which in turn phosphorylates eIF4E, enhancing its interaction with the mRNA 5’ cap (Malka-Mahieu et al., 2017; Shi et al., 2015). On the other hand, the binding of 4E-BPs to eIF4E, while it does not alter eIF4E affinity for the mRNA 5’ cap, does prevent the association between eIF4E and eIF4G, thereby inhibiting the assembly of a functional eIF4F complex. This interaction between 4E-BPs and eIF4E is regulated by phosphorylation: a hypophosphorylated form of 4E-BP1 tightly binds to eIF4E, while a hyperphosphorylated 4E-BP1 avoids this binding. The PAM pathway activation ultimately leads to the phosphorylation of 4E-BPs

by mTORC1 (Malka-Mahieu et al., 2017; Raught & Gingras, 1999; Sonenberg & Hinnebusch, 2009a).

Given the crucial role of the aforementioned proteins in regulating key cellular processes, their altered activity results in enhanced cell growth, differentiation, and survival, which ultimately lead to cancer. Mutations in RAS genes or their effectors are present in over one-third of all human malignancies. The most prevalent cancer-associated missense mutations in *RAS* genes are located at three RAS mutational hotspots: codons Gly¹², Gly¹³, and Gln⁶¹ (commonly referred to as G12, G13, and Q61, respectively), which together account for more than 90% of the point mutations observed in these genes. Mutations at these conserved residues result in constitutive RAS activation by impairing its ability to hydrolyze GTP (Huynh et al., 2022).

The RAF/MEK/ERK pathway has been reported to be among the most dysregulated pathways in human cancer (McKay & Morrison, 2007). Within this pathway, genomic alterations show a progressive decline in frequency as one moves downstream along the signaling cascade, with *RAF* mutations found in approximately 7% of human tumors, *MEK* mutations in less than 1%, and *ERK* mutations being exceedingly rare (Yaeger & Corcoran, 2019). Similarly, mutations or altered expression of components within the PAM cascade are closely associated with oncogenesis. Indeed, this pathway represents the most frequently activated signaling axis in human cancers, with approximately 50% of tumors harboring aberrations within it (Martini et al., 2014). Beyond alterations in its core effectors or regulators, dysregulation of downstream effectors of this pathway has also been implicated in carcinogenesis. One such effector is 4E-BP1, encoded by the *EIF4EBP1* gene, which acts as a key translational repressor. To date, no recurrent mutations in the *EIF4EBP1* gene have been directly associated with cancer. However, multiple studies have demonstrated that the functional dysregulation of 4E-BP1, particularly through hyperphosphorylation, is involved in oncogenesis. Supporting this, melanomas (O'Reilly et al., 2009), breast (Rojo et al., 2007; Zhou et al., 2004), prostate (Graff et al., 2009; Kremer et al., 2006), colon (Armengol et al., 2007), ovarian (Castellvi et al., 2006), and endometrial (Castellvi et al., 2009) cancers have shown a correlation between phosphorylated 4E-BP1 and increased tumor aggressiveness, as well as poor clinical outcomes. Additionally, loss of 4E-BP1 expression has been reported in pancreatic tumors (Martineau et al., 2014).

Collectively, these findings highlight the central role of RAS signaling and its downstream RAF/MEK/ERK and PI3K/AKT/mTOR pathways in tumorigenesis. Given

their strong implication in cancer progression and translational control, understanding how these signaling networks are regulated *in vivo* remains highly relevant for the identification of new therapeutic vulnerabilities. To address this, we used transgenic zebrafish models expressing fluorescent oncogenic RAS specifically in myeloid cells, allowing real-time visualization of oncogene-expressing tumor cells during leukemogenesis. Using this system, we observed an unexpected phenomenon in which oncogene-expressing neutrophils progressively disappeared during development, ultimately leading to loss of detectable oncogenic RAS expression. Hereafter, we refer to this process as oncogene silencing. We therefore investigated the molecular mechanisms underlying this phenomenon and their potential relevance to translational control and tumor suppression pathways associated with RAS-driven leukemogenesis.

MATERIALS AND METHODS

Ethics Statement

All experiments involving zebrafish complied with institutional, national, and international guidelines for animal welfare and experimentation. Experiments conducted in Murcia, Spain, adhered to the European Union Council Directive 2010/63/EU and the Spanish Royal Decree 53/2013. All procedures were approved by CARM, under protocol codes A13211202 and A13180602.

Zebrafish husbandry and general maintenance

Zebrafish (*Danio rerio* H., order Cypriniformes, family Cyprinidae) strains were obtained from the Zebrafish International Resource Center (ZIRC) and were bred, staged, and handled following established protocols (Detrich et al., 2009; Westerfield, 2000). Embryos and larvae were raised in egg water containing methylene blue at 28.5 °C. Egg water was prepared by dissolving Instant Ocean® Aquarium Sea Salt Mixture (Instant Ocean Spectrum Brands, Blacksburg, VA, USA) at 1.5 mL of a 40g/L stock per liter of distilled water (yielding a final concentration of ~60 mg/L) and supplemented with 0.5 mg/L methylene blue, following standard zebrafish husbandry protocols (Westerfield, 2007). Rotifers were introduced to the diet starting at 5 days post-fertilization (dpf), and live *Artemia* were provided from 11 dpf onward. Adult zebrafish were maintained in a recirculating aquarium system at 28 °C under a 14/10 hour light/dark photoperiod and fed

twice daily with Gemma Micro (Skretting) and live *Artemia* (MC 450, INVE Aquaculture).

The AB wild-type (WT) zebrafish strain served as the genetic background for mutant and transgenic lines. The Tg(*lyz:dsRED2*)^{nz50} line was kindly provided by Prof. Phil Crosier from the University of Auckland, New Zealand (Hall et al., 2007). In addition, the Tg(BAC*mpxo:eGFP*)ⁱ¹¹³ (also referred to as Tg(*mpx:eGFP*)) (Renshaw et al., 2006) and the TgBAC(*mpx:GAL4-VP16*)^{sh267Tg} (also known as Tg(*mpx:GAL4-VP16*)) (Davison et al., 2007) lines were generously supplied by Prof. Stephen A. Renshaw from the University of Sheffield, UK. The Tg(*UAS:eGFP-HRAS_G12V*)ⁱ⁰⁶ line was obtained from Prof. Maria Caterina Mione at the University of Trento, Italy.

The Tg(*lyz:mVENUS-NRAS_Q61L*) transgenic line was generated in our laboratory using the Gateway Tol2kit system. One-cell-stage embryos were microinjected with a vector containing a construct in which the *lyz* promoter drives the expression of an mVENUS-tagged NRAS_Q61L oncogene (a gift from Prof. Adam Hurlstone, Manchester University, UK), followed by a polyA signal. This construct was cloned into the pDestTol2cryCFP vector, which includes a lens-specific ECFP marker for screening. Tol2 transposase mRNA was co-injected to facilitate genomic integration. The remaining compound transgenic zebrafish lines used in this study were generated by crossing the aforementioned lines to achieve the desired genetic combinations. For clarity, the Tg(*xxx:xxx*)^{xxx} nomenclature will be omitted in subsequent references to transgenic lines. **Supplementary Table S1** summarizes all the transgenic lines used in this study, including the genotyping method, the labeled structure or fluorescent phenotype (e.g., green neutrophils or blue eye), and the fluorescence filter used for visualization (e.g., green, red, or blue).

CRISPR/Cas9-mediated gene editing via ribonucleoprotein microinjection

CRISPR/Cas9-mediated gene knockdown was performed according to the manufacturer's protocol. Predesigned crRNAs targeting specific genes of interest, along with the tracrRNA (#1072533) and Cas9-V3 protein (#1081058), were obtained from Integrated DNA Technologies (IDT). To form the crRNA:tracrRNA duplex, 1 µL of each RNA (100 µM) was incubated at 95 °C for 5 minutes and then allowed to cool at room temperature for 5-10 minutes. This guide RNA (gRNA) duplex was then brought to a final concentration of 50 µM by adding 1.43 µL of Duplex Buffer (IDT #11-05-01-03). The

injection mix consisted of 1 μ L of the crRNA:tracRNA complex, 0.2 μ L of Cas9 protein, 0.25 μ L of phenol red, and 2.55 μ L of Duplex Buffer. The mixture was kept on ice until use. Zebrafish embryos at the one-cell stage were microinjected into the yolk sac with 0.5-0.9 nL of the solution per embryo, using a NARISHIGE IM 300 microinjector. The same amount of gRNA were applied in every experimental group. The names and sequences of the crRNAs used in this study are listed in **Supplementary Table S2**.

To assess genome editing efficiency after microinjection, pools of approximately 30 larvae were collected at the endpoint of the experiment (7 dpf for standard experiments; 3 dpf for preliminary crRNA-testing assays). For verifying DNA conservation of the *elf4ebp1* target locus in adult zebrafish founder lines, samples consisted of adult fin clips. Genomic DNA from pooled larvae was extracted using the Wizard® Genomic DNA Purification Kit (Promega #A1125) according to the manufacturer's instructions. Genomic DNA from fin clips was extracted, the target locus was amplified by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific #2939617), following the manufacturers' protocol, and primers flanking the crRNA target site (**Supplementary Table S3**). Purified DNA was submitted for Sanger sequencing at the Genetics Platform of the Instituto Murciano de Investigación Biosanitaria (IMIB) and the resulting chromatograms were analyzed using both the TIDE (Tracking of Indels by Decomposition, <https://tide.nki.nl/>) and ICE (Inference of CRISPR Edits, <https://ice.editco.bio/#/>) algorithms to quantify genome editing efficiency.

***elf4elc* overexpression in zebrafish**

The coding sequence of *elf4elc* (NM_001017851.2), including both the WT and S205A variant, was synthesized by GenScript. RNA transcripts were generated *in vitro* using the mMESSAGE mMACHINE™ T7 Transcription kit (Ambion #AM1344), and polyadenylated using the Poly(A) Tailing kit (Invitrogen, Thermo Fisher Scientific #AM1350), following the manufacturers' guidelines. As a control, *in vitro*-transcribed enhanced GFP (EGFP) mRNA was used (Yuan & Sun, 2009). The RNAs were then mixed with microinjection buffer (0.5× Tango buffer and 0.05% phenol red solution) and injected into the yolk sac of one-cell-stage zebrafish embryos using a NARISHIGE IM 300 microinjector. A total of 200 pg of mRNA per embryo was delivered in a volume of 0.5-0.9 nL. Equal amounts of RNA were injected across all experimental groups. To

verify overexpression, total RNA was extracted from each experimental condition, and *eif4e1c* transcript levels were assessed by RT-qPCR.

To enable neutrophil-specific overexpression of either WT or mutant (S205A) zebrafish *eif4e1c*, synthetic cDNA fragments encoding each variant were obtained from GenScript in the pBluescript II KS(+) vector. These fragments were excised from the pBluescript II KS(+) vector using EcoRI and XhoI (New England Biolabs #R0101 and #R0146) and subsequently subcloned into the Gateway™ pENTR™ 1A Dual Selection Vector (Invitrogen, Thermo Fisher Scientific #A10462), previously linearized with the same enzymes. Recombinant clones were screened by ApaI digestion (New England Biolabs #R0114) and agarose gel electrophoresis to confirm correct insertion, and positive clones were verified by Sanger sequencing. Following sequence verification, Gateway™ LR recombination was performed using LR Clonase™ II enzyme mix (Invitrogen, Thermo Fisher Scientific #11791-020) to combine the 5' entry clone p5E-LyzC (Addgene plasmid #135200, containing the neutrophil-specific *lyz* promoter), the middle entry clone (pENTR™ 1A containing *eif4e1c*), and the 3' entry clone pDONR P2R-P3-SV40 polyA (Addgene plasmid #48627), into the Tol2-based destination vector pDestTol2CG2 (Tol2Kit #395). This reaction resulted in the expression construct *lyz:eif4e1c*, containing the *eif4e1c* cDNA under the control of the *lyz* promoter, followed by an SV40 polyadenylation signal and a *cm1c2:EGFP* cassette as a cardiac-specific marker for transgenesis screening. These constructs encoding *lyz:eif4e1c* (WT or S205A) were injected into one-cell-stage zebrafish embryos. Plasmid DNA (250 ng/μL) was co-injected with *in vitro*-transcribed Tol2 transposase mRNA (250 ng/ μL), in the same injection buffer described above. The total injection volume was 0.5-0.9 nL per embryo. Embryos positive for transgene integration were screened based on cardiac EGFP expression driven by the *cm1c2* promoter.

RNA extraction, cDNA synthesis, and real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from pools of 3-dpf zebrafish larvae (approximately 30 larvae per sample) using TRIzol™ reagent (Invitrogen, Thermo Fisher Scientific #15596026) in combination with the Direct-zol™ RNA Miniprep kit (Zymo Research #R2052), following the manufacturers' protocols. For WKM from individual adult fish, RNA extraction was initiated with TRIzol™, followed by purification using the RNAqueous™ Micro Total RNA Isolation Kit (Invitrogen, Thermo Fisher Scientific

#AM1931), according to the manufacturer's instructions. Genomic DNA was removed by DNase I treatment (1 U/μg RNA; Invitrogen, Thermo Fisher Scientific #222461).

First-strand cDNA synthesis was performed using the SuperScript™ IV VILO™ Master Mix (Invitrogen, Thermo Fisher Scientific #11756050), following the manufacturer's guidelines. Quantitative real-time PCR (RT-qPCR) was carried out on a StepOnePlus™ Real-Time PCR system (Applied Biosystems) using TB Green® Premix Ex Taq™ (Takara #RR420A). The cycling protocol included an initial denaturation at 95 °C for 30 seconds (s), followed by 40 amplification cycles (95 °C for 5 s, 60 °C for 20 s), and a final melting curve analysis (95 °C for 15 s, 60 °C for 1 minute, and 95 °C for 15 s).

Expression levels of target genes were normalized to the ribosomal protein S11 (*rps11*) mRNA using the comparative Ct method ($2^{-\Delta C_t}$) (Livak & Schmittgen, 2001). All qPCR experiments were performed using three independent biological replicates for larval samples, each consisting of pools of 30 larvae, with each sample run in technical triplicate. For adult zebrafish, individual WKM were analyzed separately, with each WKM representing a single biological replicate, also run in technical triplicate. Primer sequences are listed in **Supplementary Table S4**.

Single-cell RNA sequencing (scRNA-seq)

Single-cell RNA sequencing (scRNA-seq) was performed once using a pool of 30 larvae per condition. Eight-dpf *mpx:eGFP-HRAS* and *mpx:eGFP* larvae were mechanically and enzymatically dissociated using Liberase (Roche #05401119001) and then filtered through a 40 μm nylon mesh filter (Corning Falcon™ #352235), centrifuged at 500 ×g for 5 minutes at 4 °C, and resuspended in PBS containing 0.04% BSA. Cell viability (>85%) was confirmed using Trypan Blue exclusion.

Approximately 10,000 cells per sample were loaded onto a 10x Genomics Chromium Controller. cDNA libraries preparation, including reverse transcription, cDNA amplification, and sample indexing, were carried out by the Genetics and Proteomics Service of ACTI at the University of Murcia using the Chromium Single Cell 3' version 2 chemistry (10x Genomics), following the manufacturer's instructions. Final library quality was confirmed using a TapeStation (Agilent Technologies), and concentrations were measured with a Qubit Fluorometer (Thermo Fisher Scientific).

Sequencing was performed on an Illumina NovaSeq 6000 with a target depth of 50000 reads per cell. Raw data were processed using Cell Ranger (version 6.1) for demultiplexing, alignment to the *Danio rerio* GRCz11 reference genome, and count matrix generation. Downstream analyses were conducted in R using the Seurat package (version 4.3.0.1) (Satija et al., 2015). The gene count matrices obtained from Cell Ranger were imported into R using the CreateSeuratObject function, applying thresholds of min.cells = 25 to retain genes expressed in at least 25 cells, and min.genes = 250 to include cells with a minimum of 250 detected genes. Quality control filtering was subsequently applied to retain cells with more than 250 and fewer than 2500 detected genes, fewer than 15000 total UMIs, and less than 10% mitochondrial reads (based on 13 mitochondrial genes). To identify and remove potential cell doublets from the scRNA-seq dataset, the DoubletFinder method was applied (Chung & Storey, 2015), using an expected doublet rate of 7.5%

Normalization and variance stabilization were performed on the filtered data using the SCTransform function, which applies a regularized negative binomial regression model (Hafemeister & Satija, 2019; Ratnayake et al., 2021). The resulting dataset was then scaled. Principal Component Analysis (PCA)-based dimensionality reduction was executed via the RunPCA function, followed by clustering with FindNeighbors and FindClusters, using a resolution parameter of 0.5. For further dimensionality reduction and visualization, both Uniform Manifold Approximation and Projection (UMAP) and t-distributed Stochastic Neighbor Embedding (t-SNE) embeddings were computed using the RunUMAP and RunTSNE functions, based on the first 20 principal components (reduction.use = "pca", dims = 1:20).

To annotate the identified cell clusters, differential gene expression analysis was carried out using the FindAllMarkers function, after RNA expression values were normalized using the NormalizeData function and scaled using ScaleData, and applying the Wilcoxon rank-sum test with Bonferroni correction for multiple testing and setting min.pct = 0.2. Visualizations, including UMAP and feature plots, were generated using the DimPlot and FeaturePlot functions, respectively. Additional dot plots and bar plots were created using the ggplot2 package (T. Wu et al., 2021). The neutrophil cluster was identified based on the expression of canonical markers (*mpx*, *lyz*), extracted using the subset function, and reprocessed through the same normalization, clustering, and visualization pipeline as the full dataset to resolve transcriptomic heterogeneity.

Pharmacological treatments

mpx:eGFP-HRAS larvae were treated with 1 μ M XAV-939 (dissolved in 1% DMSO) from 3 to 24 dpf, with 1% DMSO alone serving as the vehicle control. During treatment, larvae were maintained in 6-well plates at 28 °C, with 15 larvae per well until 5 dpf, after which the density was reduced to 5 larvae per well to prevent overcrowding. Treatment solutions were refreshed every 24 hours. Neutrophils in XAV-939-treated larvae, along with their DMSO controls, were quantified by fluorescence imaging at 5, 7, 10, 12, 14, 17, 19, 21, and 24 dpf.

mpx:eGFP and *mpx:eGFP-HRAS* larvae were treated with 1 or 10 μ M rapamycin in 0.2% DMSO from 2 to 7 dpf. Larvae treated with 0.2% DMSO alone served as vehicle controls. During treatment, larvae were maintained in 6-well plates at 28 °C, with 15 larvae per well until 5 dpf, after which the density was reduced to 5 larvae per well. Treatment solutions were refreshed every 24 hours. Neutrophils in rapamycin-treated and vehicle-treated larvae were quantified by fluorescence imaging at 3, 5, and 7 dpf (see below).

In vivo imaging and quantification of neutrophils and fluorescence intensity in zebrafish larvae

Zebrafish larvae aged 3 to 14 dpf, from the transgenic lines *mpx:eGFP;lyz:dsRED*, *lyz:mVENUS-NRAS;mpx:eGFP;lyz:dsRED*, and *mpx:eGFP-HRAS;lyz:dsRED*, were anesthetized with buffered tricaine at 0.16 mg/mL prepared in egg water. *In vivo* imaging and image acquisition were conducted using a fluorescence stereomicroscope (Leica M205 FCA) equipped with a digital camera (Leica DFC 365 FX) and configured with green and red fluorescence filters. Neutrophils, identified as eGFP- and dsRED-positive cells, were manually counted within a defined region of the tail, extending from the cloaca to the tail tip, using the Leica Application Suite (LAS X) software.

Flow cytometry analysis of whole kidney marrow cells

Flow cytometry analyses were performed using whole kidney marrow (WKM) from adult zebrafish, with each sample representing a single adult. Three independent experiments were conducted using different zebrafish lines and age groups. In the first experiment, WKM were collected from *mpx:eGFP* and *mpx:eGFP-HRAS* adult fish aged 3 months post-fertilization (mpf). For the second experiment, WKM were obtained from *mpx:eGFP* and *lyz:mVENUS-NRAS* fish aged 23 mpf. The third experiment involved WT

(10 mpf) and *lyz:mVENUS-NRAS* (9 mpf) fish, from which WKM were also harvested for analysis.

Fish were anesthetized with tricaine and for WKM isolation, a ventral midline incision was made, and the tissue was carefully dissected and transferred into chilled fish buffer composed of 90% phosphate-buffered saline (PBS) and 5% fetal bovine serum (FBS) (Cytiva #SV3016003). Single-cell suspensions from individual WKMs were prepared by gentle pipetting and teasing through a 40 μ m nylon mesh filter (Corning Falcon™ #352340). Cells were subsequently washed and resuspended in cold fish buffer. All samples were kept on ice until flow cytometry acquisition. To exclude dead cells, propidium iodide (PI) (Molecular Probes™ #P3566) was added to a final concentration of 1 μ g/mL immediately before flow cytometry analysis.

Flow cytometric data were acquired on a BD LSRFortessa™ X-20 cytometer (BD Biosciences), gating live cells based on PI exclusion, forward scatter (FSC), and side scatter (SSC) parameters. Data analysis was performed using BD FACSDiva software, version 8.0.2 (BD Biosciences).

Statistical analyses

All statistical analyses and data visualization were performed using GraphPad Prism version 9.0. Prior to any statistical testing, all data were assessed for normality using the Shapiro–Wilk test and Kolmogorov–Smirnov test. Based on the distribution of the data, parametric or non-parametric tests were applied accordingly.

For comparisons between two groups, data with normal distribution were analyzed using an unpaired Student's *t*-test (or unpaired *t*-test). If normality was not met, a non-parametric Mann–Whitney test was considered, although it was not ultimately required in any of the analyses.

For comparisons involving more than two groups, data with normal distribution were analyzed using one-way ANOVA. Depending on the experimental design, post hoc multiple comparisons were performed using Tukey's test (when comparing all groups with each other), Dunnett's test (when comparing each group to a single control), uncorrected Fisher's LSD test (when comparing specific group pairs without correction for multiple comparisons), or Šídák's multiple comparisons test (when comparing specific group pairs with correction for multiple comparisons). In the latter case, the “do not correct for multiple comparisons” option was used in GraphPad Prism to avoid unwanted

influence between unrelated comparisons. If data were not normally distributed, a Kruskal–Wallis test was applied followed by either Dunn’s multiple comparisons test (for all-vs-all comparisons) or uncorrected Dunn’s test (for selected group comparisons without correction for multiple testing), depending on the analysis goals.

For time-course or kinetic experiments involving multiple time points, data were analyzed using two-way ANOVA. Post hoc comparisons were performed using Tukey’s test.

All experiments were independently repeated at least two or three times (N), with multiple samples included in each replicate (n), unless otherwise specified. For each condition, larvae from independent experiments were pooled for both plotting and statistical evaluation. Data are presented as mean $\pm$ standard error of the mean (SEM) or standard deviation (SD). A p-value below 0.05 was considered statistically significant. The total number of larvae per condition, the specific statistical test applied, and additional experimental details are indicated in the corresponding figure legend.

RESULTS

HRAS-driven myeloid leukemia in zebrafish undergoes spontaneous oncogene silencing during development

To study myeloid leukemia, we established a zebrafish model based on oncogenic RAS activation through the generation of distinct transgenic lines in our laboratory. To provide a baseline for comparison, we used a healthy control, the double transgenic line Tg(*mpx:eGFP*);Tg(*lyz:dsRED*), hereafter referred to as *mpx:eGFP;lyz:dsRED*, which expresses green and the red fluorescent proteins under the control of the neutrophil-specific myeloperoxidase (*mpx*) and lysozyme (*lyz*) promoters, respectively (**Figure 1Aa**). Building on this baseline, our myeloid leukemia model, Tg(*mpx:GAL4;UAS:eGFP-HRAS_G12V*), is a transgenic line expressing eGFP fused to the oncogenic HRAS_G12V, under the control of the *mpx* promoter. This line was generated using the UAS/GAL4 binary system, by crossing the Tg(*mpx:GAL4-VP16*) driver line with the Tg(*UAS:eGFP-HRAS_G12V*) effector line. For simplicity, these parental lines will be referred to as *mpx:GAL4* and *UAS:eGFP-HRAS*, with the resulting progeny as *mpx/eGFP-HRAS*.

To enable accurate visualization and comparison of neutrophils, we introduced and additional fluorescent reporter. Specifically, the *UAS:eGFP-HRAS* line was crossed with *Tg(lyz:dsRED)*, which expresses dsRED2 under the *lyz* promoter, prior to crossing with the *mpx:GAL4* driver line. This strategy enabled the simultaneous visualization of neutrophils under both *mpx* and *lyz* promoters in *mpx/eGFP-HRAS;lyz:dsRED* larvae (**Figure 1Ba**).

The fluorescent labeling of neutrophils in these transgenic lines enabled direct visualization of their distribution and facilitated tracking of myeloid leukemia progression *in vivo*. Fluorescent images of larvae were acquired using a fluorescence stereo microscope. In control *mpx:eGFP;lyz:dsRED* larvae, neutrophils (dsRED⁺ and eGFP⁺ cells) were mainly restricted to the caudal hematopoietic tissue (CHT) and the developing kidney (**Figure 1Ac**). In contrast, larvae from the HRAS-driven myeloid leukemia model showed oncogenic expansion and dispersal of neutrophils throughout the body, with dsRED⁺ cells enriched in the CHT (**Figure 1Bc**).

To monitor myeloid leukemia progression over time, we quantified fluorescent neutrophils in the tail region of zebrafish larvae from 2 to 25 days post-fertilization (dpf). Comparison of eGFP⁺ neutrophil numbers between *mpx/eGFP-HRAS* and *mpx:eGFP* larvae revealed a marked expansion of neutrophils in *mpx/eGFP-HRAS* larvae from 2 to 7 dpf, reaching a peak at 7-8 dpf, followed by a progressive decline in eGFP⁺ neutrophil numbers. In contrast, eGFP⁺ neutrophil numbers in *mpx:eGFP* larvae remained relatively stable, with a pronounced increase from 15 dpf onward (**Figure 1C**).

Next, focusing specifically on the leukemic background, we analyzed *mpx/eGFP-HRAS* larvae at 15, 20, and 25 dpf, quantifying both eGFP⁺ and dsRED⁺ neutrophils. Over this period, eGFP⁺ neutrophils continued their gradual decline, while dsRED⁺ neutrophils increased concomitantly (**Figure 1D, E**), similar to the pattern observed in healthy controls (**Figure 1C**). This ultimately resulted in the complete absence of eGFP⁺ neutrophils in adult fish. This phenomenon, characterized by eGFP⁺ neutrophil elimination, will hereafter be referred to as “oncogene silencing”.

Given the absence of eGFP⁺ cells in adult fish, we next sought to determine whether this was associated with broader alterations in hematopoiesis. To this end, whole kidney marrow (WKM) cell populations were examined by flow cytometry. WKM cell suspensions from 12 *mpx/eGFP-HRAS* fish at 3 months post-fertilization (mpf) were analyzed and compared to those from 12 age-matched *mpx:eGFP* control fish (**Figure 1F**). No statistically significant differences were observed in the proportions of myeloid,

erythroid, lymphoid, or precursor populations (**Figure 1G**). Importantly, no expansion of the myeloid compartment was detected, consistent with the absence of overt leukemia in adult fish.

A second RAS-driven myeloid leukemia model, *lyz:mVENUS-NRAS*, reveals transient larval neutrophilia that does not translate into adult leukemic progression.

Based on these results, we generated a second, independent RAS-driven myeloid leukemia model to test the generality of these findings: Tg(*lyz:mVENUS-NRAS_Q61L*), designated *lyz:mVENUS-NRAS*, which expresses a fusion of the fluorescent protein mVENUS with the oncogenic NRAS_Q61L, driven by the *lyz* promoter. Notably, the mVENUS-NRAS fusion protein did not produce a detectable fluorescence signal. To overcome this limitation, the line was crossed with the double transgenic line Tg(*mpx:eGFP*);Tg(*lyz:dsRED*) (**Figure 1A**), as schematically illustrated in **Supplementary Figure S1Aa**. Fluorescence stereomicroscopy revealed that *lyz:mVENUS-NRAS;mpx:eGFP;lyz:dsRED* larvae displayed systemic neutrophil expansion and dissemination (**Supplementary Figure S1Ac**), recapitulating the phenotype observed in the HRAS-driven myeloid leukemia model (**Figure 1Bc**).

We next monitored neutrophil expansion as a functional readout of transgene activity to assess whether this alternative promoter–oncogene combination drives a more consistent or sustained myeloid transformation. Quantification of eGFP⁺ neutrophils from 3- to 14-dpf *lyz:mVENUS-NRAS;mpx:eGFP* larvae compared to *mpx:eGFP* controls showed an initial expansion peaking at 7 dpf, followed by a transient decline and a subsequent increase in neutrophil numbers (**Supplementary Figure S1B**). This dynamic resembles the temporal profile observed in *mpx/eGFP-HRAS* larvae.

To determine whether this early NRAS-driven expansion persists in adulthood, we next examined adult fish. Throughout routine maintenance, adult fish from the *lyz:mVENUS-NRAS* line showed no overt signs of illness or increased mortality. To assess whether these fish developed myeloid leukemia, we collected WKM samples from six *lyz:mVENUS-NRAS* fish and six *mpx:eGFP* control fish at 23 mpf. To capture the range of phenotypic variability within the *lyz:mVENUS-NRAS* group, we selected two individuals in poor physical condition (marked in red in the graphs), two with intermediate appearance (orange), and two apparently healthy fish (green) (**Supplementary Figure S1C**). Flow cytometry analysis was performed to quantify the

percentage of myeloid cells (**Supplementary Figure S1D**), revealing no statistically significant differences between both genotypes (**Supplementary Figure S1E**).

Given the absence of a leukemic phenotype in adult *lyz:mVENUS-NRAS* fish by flow cytometry, we investigated whether they still expressed the *mVENUS-NRAS* fusion transcript in their WKM. First, a primer pair targeting the fusion site was designed for RT-qPCR detection (**Supplementary Table S4**). Despite suboptimal efficiency for absolute quantification, the primers consistently amplified the transcript, allowing for relative, though not strictly quantitative, expression comparisons across samples. We next performed RT-qPCR on WKM samples from the 12 23-mpf adult fish (six *lyz:mVENUS-NRAS* and six *mpx:eGFP*) previously analyzed by flow cytometry. Pooled 6-dpf *lyz:mVENUS-NRAS* larvae were included as a positive control. The fusion transcript was detected in the larval sample and in four of the six *lyz:mVENUS-NRAS* adults analyzed (fish 2–5), but not in *mpx:eGFP* controls or in the remaining two *lyz:mVENUS-NRAS* fish (the ones with the worst (fish 1) and best (fish 6) physical condition, **Supplementary Figure S1B**). Expression levels in the four positive adults were markedly reduced, ranging from 0.007 to 0.048 relative to the larval control (set as 1), indicating minimal fusion transcript expression in adult WKM (**Supplementary Figure S1F**).

We next evaluated whether earlier signs of myeloid leukemia could be detected at a younger age. To this end, we performed the same flow cytometry analysis on WKM samples from 13 WT control fish at 10 mpf, and from 12 *lyz:mVENUS-NRAS* fish at 9 mpf. This analysis again revealed no statistically significant differences in myeloid cell percentages between both groups (**Supplementary Figure S1G**).

Taken together, these results indicate that *lyz*-driven expression of oncogenic *NRAS* induces a transient neutrophil expansion in larvae but is insufficient to drive leukemic progression in adulthood, likely due to low transgene expression levels.

scRNA-seq reveals transcriptional heterogeneity and HRAS-dependent neutrophil states during oncogene silencing

To investigate the molecular mechanisms underlying oncogene silencing, we performed a single-cell RNA sequencing (scRNA-seq) on *mpx:eGFP-HRAS;lyz:dsRED* larvae at 8 dpf, the time point at which the number of eGFP⁺ neutrophils begins to decline. This approach aimed to identify transcriptional changes associated with HRAS-driven

neutrophils and to uncover potential cellular states or molecular pathways involved in this silencing process.

The scRNA-seq dataset was initially clustered into 24 transcriptionally distinct populations based on gene expression profiles (**Figure 2A**). Among these, cluster 18 was identified as the neutrophil population based on the high expression of the canonical neutrophil markers *lyz* and *mpx* (**Figure 2B**). This neutrophil cluster was subsequently re-clustered to resolve transcriptomic heterogeneity among its constituent cells, yielding six transcriptionally distinct subclusters (**Figure 2C**). For consistency with standard scRNA-seq conventions, we maintained the default numerical cluster labels (0-5), as these do not imply any biological hierarchy or sequential order. Marker genes defining each neutrophil subcluster are listed in **Data Set 1**.

As expected, the expression of *lyz* and *mpx* was broadly distributed across all neutrophil subclusters (**Figure 2D**), confirming their shared neutrophil identity, with subclusters 3 and 5 appearing to express *lyz* at somewhat higher levels. We then examined *HRAS* expression within these subclusters. Unlike *lyz* and *mpx*, *HRAS* expression was heterogeneous, enriched in subclusters 0, 1, 2, and 4, while subcluster 3 showed low expression and subcluster 5 no detectable expression, consistent with a less oncogenically activated transcriptional state (**Figure 2E**). Finally, differential gene expression analysis between *HRAS*⁺ and *HRAS*⁻ neutrophils (see **Data Set 2** for the full list) revealed two genes of particular interest due to their involvement in the transcriptional (*ctnnb1*) and translational (*EIF4EBP1*) regulation of protein synthesis, including RAS: *ctnnb1*, predominantly expressed in subclusters 1 and 4, and *EIF4EBP1*, selectively enriched in subcluster 1 (**Figure 2F**).

These findings prompted us to functionally investigate the contribution of *ctnnb1* and *EIF4EBP1* to oncogene silencing *in vivo*.

Inhibition of the Wnt/ β -catenin pathway does not affect neutrophil dynamics in *mpx/eGFP-HRAS* larvae

To investigate whether *ctnnb1*, the gene encoding β -catenin, plays a functional role in mediating oncogene silencing in this model, we treated *mpx/eGFP-HRAS* larvae with the Wnt/ β -catenin pathway inhibitor XAV-939 (Al-Hamaly et al., 2024; Robertson et al., 2014) at 3 dpf (schematic of the pathway and inhibitor target site is shown in **Figure 3A**). eGFP⁺ neutrophils were quantified in the tail region of treated larvae from 5 to 24

dpf and compared to control *mpx/eGFP-HRAS* siblings treated with DMSO at the same developmental stage (**Figure 3B**). Representative images of the tail region of 10- and 12-dpf larvae from both groups are shown in **Figure 3C**. Statistical analysis of quantified eGFP⁺ neutrophils revealed no significant differences in neutrophil number between XAV-939-treated and control larvae at any time point analyzed (**Figure 3D**).

Genetic manipulation of *eif4ebp1* and *eif4e1c* reveals their roles in homeostasis and oncogene-driven leukemogenesis

Given that β -catenin signaling inhibition did not impact oncogene silencing, we next investigated the potential role of *eif4ebp1* in this process. EIF4EBP1 is a member of the eIF4E-binding protein (4E-BP) family of translational repressors. Its main function is to bind to the initiation factor eIF4E and prevent it from forming the eIF4F complex, which is necessary to initiate the translation of mRNA into proteins (**Figure 4A**). EIF4EBP1 overexpression results in enhanced repression of translation, especially of mRNAs that are highly dependent on eIF4E, such as those with long and structured 5' UTRs (Huang et al., 2009).

To this end, we employed a CRISPR/Cas9-based gene editing approach to disrupt *eif4ebp1* function in *mpx/eGFP-HRAS* larvae. This strategy allowed us to directly assess whether loss of *eif4ebp1* alters neutrophil expansion and subsequent oncogene silencing observed in our zebrafish model. A single guide RNA (crRNA) was designed to target exon 2 of the gene (**Supplementary Figure S2A**). This site was selected to induce a frameshift mutation expected to generate a loss-of-function allele. To verify DNA conservation of the target region in the zebrafish lines used (workflow shown in **Supplementary Figure S2B**), tail biopsies were collected from adult *mpx:eGFP*, *lyz:dsRED*, *mpx:GAL4* and *UAS:eGFP-HRAS* specimens employed for breeding. Genomic DNA was extracted, and the target locus was amplified by PCR using high-fidelity PfuUltra DNA polymerase and primers flanking the crRNA binding site. The PCR products were subsequently purified and sequenced. Complete conservation of the target sequence in all transgenic lines was confirmed (**Supplementary Figure S2C**).

The crRNA was then tested by microinjection into *wild-type* zebrafish embryos at the one-cell stage. Editing efficiency was assessed by Sanger sequencing of PCR amplicons from *eif4ebp1*-crRNA-injected larvae, followed by analysis using the ICE (Inference of CRISPR Edits) algorithm, which revealed efficient cleavage and indel

formation at the target site (**Supplementary Figure S2D**). The designed crRNA showed editing efficiencies ranging from 76% to 81% and knockout scores from 50 to 70, indicating substantial loss-of-function potential. This validation confirmed the suitability of the crRNA for downstream functional studies.

After validating the *EIF4EBP1*-targeting crRNA, it was employed in *mpx:eGFP-HRAS;lyz:dsRED* larvae to examine the role of this gene in oncogene silencing (**Figure 4B**, top panels). One-cell stage embryos were microinjected with CRISPR/Cas9 components along with either *EIF4EBP1*-targeting or control (scramble) crRNAs, and neutrophil numbers were quantified in the tail region from 5- to 12-dpf larvae. Statistically significant increases in eGFP⁺ neutrophils were observed at 5, 7, and 12 dpf in *EIF4EBP1*-targeted larvae (**Figure 4C**), while dsRED⁺ neutrophil numbers remained unchanged compared to controls throughout the analyzed period, except for 7 dpf (**Figure 4D**), suggesting impaired oncogene silencing.

The same approach was applied to healthy *lyz:dsRED* larvae (**Figure 4B**, bottom panel). In these larvae, *EIF4EBP1* disruption led to a transient increase in neutrophil numbers at 7 dpf, followed by a significant decrease at 10 dpf compared to controls. A similar downward trend was observed at 12 dpf, although this difference did not reach statistical significance (**Figure 4E**).

Given the significant effect of *EIF4EBP1* disruption on eGFP⁺ neutrophil counts in 7-dpf *mpx:eGFP-HRAS* larvae, we investigated whether targeting its binding partner Eif4e1c (**Figure 5A**) would elicit an opposite effect. To disrupt *EIF4E1C*, two CRISPR/Cas9 crRNAs (*EIF4E1C* crRNA1 and *EIF4E1C* crRNA2) targeting different regions of the gene were designed and co-injected to increase editing efficiency. The phenotypic effects of *EIF4E1C* disruption were then evaluated in a comparative analysis. One-cell stage embryos from *mpx:eGFP-HRAS* and *mpx:eGFP* lines were microinjected with CRISPR/Cas9 components targeting either (1) a control (non-targeting) sequence, (2) *EIF4EBP1*, or (3) *EIF4E1C*. Neutrophil numbers were quantified at 7 dpf by counting eGFP⁺ cells in the tail region. Because only eGFP⁺ neutrophils were analyzed, 7-dpf *mpx:eGFP* larvae were used as healthy controls (**Figure 5A**). In the *mpx:eGFP-HRAS* background, targeting *EIF4EBP1* resulted in increased neutrophil numbers compared to the control group, as previously shown, whereas disruption of *EIF4E1C* led to a significant decrease in eGFP⁺ neutrophils relative to controls (**Figures 5B, 5C**). These results underscore the

importance of the Eif4ebp1-Eif4e1c interaction in the oncogene silencing observed in this model.

In the *mpx:eGFP* background, a significant reduction was observed only in *EIF4E1C*-targeted larvae compared to controls, suggesting a possible role for this gene in myelopoiesis. Neutrophil numbers in the *EIF4EBP1*-targeted group did not differ significantly from controls (**Figures 5D, 5E**), although a slight downward trend was observed, consistent with the decrease seen in *lyz:dsRED* larvae at later stages (**Figure 4G**). Editing efficiency was confirmed by Sanger sequencing followed by ICE analysis (**Supplementary Figure S3A**).

To further investigate the functional role of *EIF4E1C*, we adopted a complementary gain-of-function approach by overexpressing the gene. One-cell stage embryos from both *mpx:eGFP* and *mpx/eGFP-HRAS* lines were microinjected with synthetic mRNA encoding either (1) GFP (control), (2) WT Eif4e1c, or (3) a non-phosphorylatable Eif4e1c mutant (S205A) (**Figure 6A**). In both lines, quantification of eGFP⁺ neutrophils in the tail region at 3 dpf revealed that injection of WT Eif4e1c mRNA significantly increased neutrophil numbers compared to the control, whereas the S205A mutant failed to increase neutrophil numbers and even showed a trend toward reducing them, reaching significance in the *mpx:eGFP* line (**Figures 6B–6E**). To verify the efficiency of mRNA overexpression, we performed RT-qPCR on pooled 3-dpf larvae from each injection group. As expected, both mRNA injections resulted in markedly increased *EIF4E1C* transcript levels compared to the GFP control, confirming successful overexpression (**Supplementary Figure S3B**).

Based on the results obtained in the *mpx/eGFP-HRAS* model and the observation of a similar silencing phenotype in the *lyz:mVENUS-NRAS* background, we next assessed the effect of *EIF4E1C* overexpression in this second model using a neutrophil-specific approach. To this end, Tol2-based plasmids were generated in which the *EIF4E1C* coding sequence, either WT or the S205A mutant, was placed under the control of the *lyz* promoter. To obtain initial functional insights, mosaic overexpression was induced by microinjecting these plasmids at the one-cell stage into *lyz:mVENUS-NRAS;mpx:eGFP* embryos (**Figure 6F**). Neutrophils were subsequently quantified in the tail region at 5, 7, and 12 dpf by fluorescence imaging (**Figure 6G**). This analysis revealed that mosaic overexpression of WT *EIF4E1C* led to an increased number of eGFP⁺ neutrophils, whereas

injection of the plasmid carrying the mutant variant did not significantly affect neutrophil numbers compared to uninjected controls (**Figure 6H**).

Pharmacological inhibition of Mtor potentiates oncogene silencing in HRAS-driven leukemogenesis

Building on these findings, and given that Mtor-mediated phosphorylation negatively regulates Eif4ebp1 activity, we next investigated whether pharmacological inhibition of Mtor could enhance oncogene silencing. To test this, *mpx/eGFP-HRAS* larvae were treated with rapamycin, an inhibitor of Mtor (Saxton & Sabatini, 2017), from 2 to 7 dpf (**Figures 7A, 7B**).

To identify the most effective rapamycin concentration, *mpx/eGFP-HRAS* larvae were treated with either 1 or 10 μ M rapamycin, alongside vehicle-treated controls, and eGFP⁺ neutrophils were quantified throughout larval development. Representative images of 7-dpf larvae from the three groups are shown in **Figure 7C**. Quantification revealed significant main effects of treatment and time, indicating that both 1 and 10 μ M rapamycin markedly reduced oncogene-driven neutrophil expansion compared with DMSO-treated controls, with the strongest effect observed at 10 μ M (**Figure 7D**).

Based on these results, 10 μ M rapamycin was selected for a second independent experiment to validate its effect on oncogene-driven neutrophil expansion. Representative images of DMSO- and rapamycin-treated *mpx/eGFP-HRAS* larvae at 5 dpf are shown in **Figure 7E**. Quantification of eGFP⁺ neutrophil at 3, 5, and 7 dpf confirmed a robust reduction in eGFP⁺ neutrophil numbers at all developmental stages following rapamycin treatment, indicating that pharmacological inhibition of Mtor strongly potentiates oncogene silencing (**Figure 7F**).

Finally, to determine whether this effect was specific to the oncogenic context, *mpx:eGFP* larvae were also treated with 10 μ M rapamycin using the same experimental design. Strikingly, rapamycin treatment did not significantly alter neutrophil numbers in the *mpx:eGFP* background (**Figures 7G, 7H**).

DISCUSSION

In this work, we report a striking phenomenon during the analysis of neutrophil dynamics in *mpx/eGFP-HRAS;lyz:dsRED* larvae. In this model, following an initial expansion of eGFP⁺ neutrophils, which recapitulates the myeloid leukemia-like

phenotype driven by HRAS, we found that this expansion peaks at 7-8 dpf, after which the number of eGFP⁺ (and thus HRAS⁺) neutrophils progressively declines until none remain. Remarkably, this regression occurs spontaneously, without any experimental switch-off or immune manipulation. Studies performed in *lyz:mVENUS-NRAS;mpx:eGFP* larvae revealed temporal dynamics resembling those previously described in the *mpx/eGFP-HRAS* model. This pattern suggests that transient oncogenic activation may not be restricted to a particular oncogene (*HRAS* vs. *NRAS*) or promoter (*mpx* vs. *lyz*) but could instead represent a broader phenomenon of “oncogene silencing” in zebrafish RAS-driven myeloid leukemia-like models. An important distinction between the two models, however, is the inability to directly monitor NRAS-driven oncogenic activity. In the HRAS model, the loss of eGFP fluorescence provided direct evidence of oncogene silencing, whereas in the NRAS line the absence of detectable mVENUS signal prevents such inference. The most plausible explanation for this limitation is that the fusion of mVENUS to NRAS compromises the proper folding of the fluorophore, thereby impairing its maturation and fluorescence emission.

This interpretation is further supported by observations in adult *lyz:mVENUS-NRAS* animals, which do not display the overt myeloid expansion characteristic of myeloid leukemia, in which clonal expansion of blasts (immature myeloid-derived cells) in peripheral blood and bone marrow is a defining feature (Vakiti et al., 2025), nor do they show obvious signs of illness or increased mortality during routine maintenance. Flow cytometry analysis of WKM at 23 mpf revealed no significant differences in myeloid cell percentages compared to *mpx:eGFP* controls. Notably, this lack of myeloid expansion contrasts with other zebrafish myeloid leukemia models where adult fish do develop leukemia, such as the Tg(*lyz:hMYC*) transgenic line, in which acute myeloid leukemia (AML) is driven by the *hMYC* oncogene under the *lyz* promoter and adult WKM displays clear expansion of myeloid cells (Luciano et al., 2023).

A survey of the literature suggests that this type of gradual and complete regression is unique in the field, as previous studies typically report either sustained leukemic expansion or acute resolution (Harrison et al., 2016). Comparable phenomena have only been reported in immune-dependent spontaneous tumor regressions, such as clearance of Raji B-cell lymphomas by CD4⁺ and CD8⁺ T cells in humanized mice (Patel et al., 2023), or innate leukocyte-mediated lysis of transplanted tumors in resistant mouse colonies (Cui et al., 2003). However, these examples differ fundamentally from our observations, as they involve immune-mediated clearance of exogenous tumor grafts, whereas our model

relies on a stable transgenic line designed to express an oncogene in neutrophil progenitors, in which the oncogenic population regresses autonomously. Importantly, expression of the same oncogenic RAS variants in other zebrafish tissues has been shown to drive highly aggressive malignancies, including glioblastoma when expressed in neural progenitors (Mayrhofer et al., 2017), myeloid leukemia when expressed in hemogenic endothelium (Alghisi et al., 2013), and melanoma when targeted to melanocytes (Santoriello et al., 2010). This suggests that the spontaneous regression observed in our model is unlikely to reflect intrinsic weakness of the oncogene itself, but rather a lineage-specific suppressive mechanism operating within the neutrophil compartment. Notably, spontaneous regression has also been documented in human cancers, albeit infrequently (Cui et al., 2003), suggesting that our model may capture a biologically relevant instance of intrinsic oncogenic regression that warrants further mechanistic investigation.

We hypothesized that this intrinsic regression, here defined as “oncogene silencing,” could arise through (i) oncogene-induced senescence (OIS), a complex program triggered by aberrant activation of oncogenic signaling, classically exemplified by RAS (Liu et al., 2018), as in our model; (ii) selective elimination of transformed clones through cell competition or immune surveillance, as described previously (Ramos & Martins, 2021), whereby *HRAS*⁺ cells could be selectively lost before establishing a stable leukemic population; and/or (iii) transcriptional or proteasomal repression of the oncogene, which could contribute to reducing oncogene activity in transformed cells, as suggested by studies showing that RAS levels can be regulated by proteasomal degradation and transcriptional mechanisms involving factors such as TP53 control (Jeong et al., 2012; Spandidos et al., 1995; Wan et al., 2017; Zheng et al., 2018).

To dissect these possibilities, we profiled the transcriptional landscape of neutrophils from 8-dpf *mpx/eGFP-HRAS* larvae, the stage at which eGFP-*HRAS*⁺ cell numbers begin to decline, using single-cell RNA sequencing (scRNA-seq). Our analysis revealed pronounced transcriptional heterogeneity within the neutrophil lineage. Unsupervised clustering identified six transcriptionally distinct neutrophil subclusters, revealing that oncogenic RAS activation does not uniformly impact the myeloid compartment but instead follows a progressive trajectory of transformation. Subclusters 0, 2, 3, and 4 exhibited heterogeneous but detectable *HRAS* expression, whereas subclusters 3 and 5 displayed low (subcluster 3) to absent (subcluster 5) expression. This gradient suggests a continuum of transformation, plausibly progressing from subcluster 5 to 3 to 0/2 to 1/4, representing the sequential acquisition of oncogenic features. These

findings align with the context-dependent transcriptional heterogeneity described for neutrophils in scRNA-seq datasets (McLaren et al., 2023).

Subclusters 3 and 5, which exhibit the highest *lyz* expression, are consistent with the dsRED⁺ (*lyz*⁺) neutrophil population identified in *mpx:eGFP-HRAS;lyz:dsRED* larvae. These dsRED⁺ neutrophils resemble those from the *mpx:eGFP* healthy control line, as their numbers progressively increase in the tail region from 15 to 25 dpf. In contrast, eGFP⁺ neutrophils (HRAS-transformed, *mpx*⁺) show the opposite trend, progressively declining over the same developmental window. Spatially, dsRED⁺ neutrophils are predominantly located in the CHT, whereas eGFP⁺ neutrophils display increased dispersion. Notably, dsRED⁺ neutrophils from *mpx:eGFP-HRAS;lyz:dsRED* larvae appear less confined to the CHT compared to neutrophils in the *mpx:eGFP* line, which may reflect the presence of double-positive (dsRED⁺/eGFP⁺) cells within this population. This transcriptional-phenotypic correspondence aligns with the developmental sequence of neutrophils, where *lyz* expression precedes *mpx* activation. These findings support a model in which HRAS-driven transformation occurs selectively in a transcriptionally and functionally defined subset of more differentiated *mpx*-expressing neutrophil precursors, while the dsRED⁺/*lyz*⁺ population represents a quiescent, non-oncogenic reservoir insulated from leukemogenic reprogramming. This highlights that leukemogenesis proceeds through discrete cellular states rather than a uniform transformation of the entire lineage, as shown by single-cell transcriptome analysis in a MYC-driven AML model, which revealed a stepwise progression of leukemic states with increasing molecular heterogeneity (B. Wu et al., 2023).

Among the differentially expressed genes, *ctnnb1* and *EIF4EBP1* emerged as candidate regulators, highlighting a potential link between oncogene silencing and the regulation of protein degradation and synthesis. The enrichment of *ctnnb1* in subclusters 1 and 4 raises the possibility of Wnt/ β -catenin involvement, which aligns with one of our proposed hypotheses for oncogene silencing. Furthermore, the selective enrichment of *EIF4EBP1* in subcluster 1 points to a translational checkpoint specifically engaged in oncogene-expressing neutrophils.

To further interpret these findings, we explored the potential role of *ctnnb1* and *EIF4EBP1* in oncogene silencing by examining their associated signaling pathways. This led us to propose that oncogene silencing may proceed via two non-mutually exclusive mechanisms: (i) enhanced proteasomal degradation of the eGFP-HRAS fusion protein mediated by β -catenin, and/or (ii) translational repression of the fusion protein mediated

by Eif4ebp1 activity. To investigate the first mechanism, we focused on *ctnnb1*, which encodes β -catenin, a multifunctional protein best known as a central effector of the canonical Wnt signaling pathway (Valenta et al., 2012). β -catenin can directly interact with RAS and serve as a molecular switch for its GSK3 β -mediated proteasomal degradation (Lee et al., 2018). According to S. Lee et al. (2018), degradable wild-type β -catenin is first phosphorylated and targeted for proteasomal degradation, which in turn exposes RAS to GSK3 β phosphorylation and subsequent β -TrCP-mediated degradation. Conversely, when WT β -catenin is mutated or otherwise functionally stabilized, RAS remains “masked,” preventing its phosphorylation and protecting it from degradation. Based on this, a reduction in β -catenin levels would be expected to facilitate oncogene silencing by promoting RAS degradation through its unmasking. However, the transcriptional profile of *ctnnb1* in our scRNA-seq data, with elevated expression in certain subclusters, was not fully consistent with a primary role in neutrophil clearance and appeared paradoxical with enhanced silencing. Despite this, and given the established role of β -catenin in RAS degradation, we sought to determine whether its activity was functionally relevant in our model, specifically, whether changes in *ctnnb1* expression might modulate oncogene silencing. To this end, we pharmacologically inhibited the Wnt/ β -catenin signaling pathway using the small molecule inhibitor XAV-939 (Al-Hamaly et al., 2024; Antony et al., 2023; Robertson et al., 2014). However, no significant differences in neutrophil number were observed between treated and control larvae at any time point. These results indicate that pharmacological inhibition of β -catenin does not prevent or delay oncogene silencing, suggesting that *ctnnb1* is not critically involved in regulating this process in our model. Consistent with this, the heterogeneous and cluster-specific pattern of *ctnnb1* expression in the scRNA-seq data likely reflects a stress-associated transcriptional response rather than a primary driver of neutrophil clearance, especially given that cellular stress can induce Wnt/ β -catenin signaling and its downstream targets (e.g., ATF3) (Dannheisig et al., 2021; Inoue et al., 2018).

Since the inhibition of β -catenin did not alter the course of oncogene silencing, we turned our attention to eif4ebp1. Given that EIF4EBP1 functions as a repressor of cap-dependent translation, its upregulation in our dataset likely reflects a mechanism that primes RAS-driven transformed neutrophils for elimination once translational capacity becomes limiting. However, we cannot rule out the alternative possibility that the apparent loss of fluorescent signal results not from neutrophil elimination per se, but rather from suppression of translation of the eGFP-HRAS fusion transcript. In this

scenario, the cells might persist but become undetectable due to loss of fluorescence. Importantly, such translational silencing would also prevent expression of the oncogenic HRAS protein, implying that these cells would no longer maintain an oncogenic state, even if they remain present. Notably, our observations support this latter possibility, as larvae progressively accumulate *lyz:dsRED*⁺ neutrophils lacking eGFP-HRAS expression, which become preferentially localized within the CHT rather than displaying the widespread dispersal characteristic of oncogenic neutrophils. This behavior resembles that of non-transformed neutrophils, suggesting that at least a fraction of the oncogenic population may revert toward a more homeostatic-like state following suppression of oncogene expression.

Regardless of the precise mechanism, the *eif4ebp1* enrichment found in our dataset aligns with a growing body of evidence supporting its tumor-suppressive role. For instance, in head and neck squamous cell carcinoma (HNSCC), loss of *EIF4EBP1* gene copy number has been associated with reduced protein levels and poor patient prognosis, while restoration of its activity can suppress tumor growth. Indeed, *eif4ebp1/2*-knockout mice develop larger and more numerous lesions in models of HNSCC carcinogenesis (Wang et al., 2019). Similarly, in ovarian and breast cancers, as well as childhood rhabdomyosarcoma, elevated EIF4EBP1 levels correlate with improved survival outcomes (Sonenberg & Hinnebusch, 2009b). Taken together, these findings led us to hypothesize that genetic disruption of *eif4ebp1* could impair the oncogene silencing process or, at least, enhance the leukemic burden in our larvae. To test this, we designed and validated a CRISPR/Cas9 strategy targeting *eif4ebp1* and microinjected the components into one-cell stage embryos of both *mpx/eGFP-HRAS;lyz:dsRED* larvae and healthy *lyz:dsRED* controls. The genetic disruption of *eif4ebp1* in *mpx/eGFP-HRAS;lyz:dsRED* larvae resulted in a significant increase in the number of eGFP⁺ neutrophils at multiple timepoints, while the dsRED⁺ neutrophil population largely remained stable. This selective expansion of transformed neutrophils suggests that the loss of *eif4ebp1* impairs oncogene silencing, thereby maintaining or enhancing the leukemic phenotype. Although not statistically significant, a subtle trend toward increased dsRED⁺ neutrophil numbers was observed following *eif4ebp1* disruption. This minor elevation is likely influenced by a small subset of double-positive (eGFP⁺ dsRED⁺) neutrophils, which appear to exhibit an oncogenic status conferred by eGFP-HRAS expression. Consequently, this population may display proliferative behavior that inflates the dsRED⁺ cell count. Together, these results support a functional role for Eif4ebp1 in

regulating oncogene silencing and controlling leukemic burden in this model, reinforcing its tumor-suppressive function as previously suggested by our transcriptomic data and the literature.

The effect of *eif4ebp1* disruption in non-transformed neutrophils also suggests a potential role for this gene in supporting neutrophil expansion or survival under homeostatic conditions. This dual role underscores a context-dependent function of Eif4ebp1. These findings are consistent with previous *in vivo* murine studies on normal myelopoiesis, in which deletion of *eif4ebp1* impaired granulocytic differentiation, leading to an accumulation of immature precursors and a decrease in mature granulocytes (Olson et al., 2009). Together, these data support a conserved role for Eif4ebp1 in myelopoiesis, particularly within the granulocytic lineage, and suggest that its function in neutrophil development may be evolutionarily preserved. While these initial observations lay the groundwork for understanding the physiological roles of Eif4ebp1, further studies will be essential to assess how its deficiency affects myelopoiesis and immune function at later developmental stages and into adulthood. Once established, this model will provide a powerful platform to dissect the context-dependent roles of Eif4ebp1 in leukemic transformation and progression.

To further explore the functional relevance of the Eif4ebp1/Eif4e1c axis in leukemic and non-oncogenic neutrophils, we performed a series of transient CRISPR-based experiments targeting *eif4e1c*, the cap-binding partner of Eif4ebp1. While Eif4ebp1 acts as a repressor of cap-dependent translation by sequestering Eif4e proteins (Sonenberg & Hinnebusch, 2009b), Eif4e1c would act as a key effector within this pathway, particularly in zebrafish where it represents a broadly expressed Eif4e1 isoform (Rao et al., 2023). Although gene targeting of *eif4e1c* significantly reduced neutrophil numbers in the *mpx/eGFP-HRAS;lyz:dsRED* and *lyz:dsRED* backgrounds, *eif4ebp1* disruption increased neutrophil numbers in the leukemic context but tended to decrease them in healthy larvae. These findings indicate that, in transformed cells, there is a functional antagonism between Eif4ebp1 and Eif4e1c, where repression or activation of cap-dependent translation exerts opposing effects. Taken together, these results suggest that while Eif4ebp1 exhibits a context-dependent, dual role, supporting neutrophil homeostasis under physiological conditions and limiting leukemic expansion under oncogenic stress, Eif4e1c appears to be indispensable for neutrophil maintenance in both contexts, pointing to a broader or more fundamental role in basal myelopoiesis. This supports the notion that cap-dependent translation remains critical even in transformed

cells, not only to sustain essential hematopoietic programs, but also to maintain the expression of oncogenic drivers that fuel leukemic progression. Consistent with this, Malka-Mahieu et al. (2017) have reported that eIF4E is implicated in the translation of mRNAs that encode proteins involved in cell growth, cell-cycle progression (e.g., MYC and CCND1) or more generally cancer-related genes. Alternatively, Eif4e1c may regulate a more essential subset of mRNAs required for neutrophil survival, whereas Eif4ebp1 serves as a context-sensitive modulator of its activity.

We next complemented our loss-of-function approach by overexpressing both the WT Eif4e1c and a non-phosphorylatable S205A mutant variant in *mpx:eGFP* and *mpx/eGFP-HRAS* larvae. Serine 209 of mouse EIF4E (S205 in zebrafish) is a conserved regulatory site whose phosphorylation enhances cap-binding affinity and promotes the selective translation of specific mRNAs (Wendel et al., 2007). This post-translational modification has been associated with oncogenesis by increasing EIF4E activity, and the zebrafish S205A mutant, being unable to be phosphorylated, is therefore expected to lack full translational capacity, serving as a tool to dissect the functional importance of this modification. In both backgrounds, overexpression of WT *eif4e1c* led to a significant increase in neutrophil numbers, whereas the S205A mutant produced the opposite effect. The opposing phenotypes observed upon disruption versus overexpression of WT *eif4e1c* reinforce the specificity and robustness of this regulatory axis. Moreover, our findings are consistent with previous *in vitro* studies showing that eIF4E reduction inhibits tumor development, while its overexpression promotes malignant transformation and uncontrolled cell growth (Martínez-Díaz, 2014). In addition, the effect observed upon Eif4e1c^{S205A} overexpression underscores the critical role of S205 phosphorylation in enabling full pro-translational activity and aligns with studies reporting the inability of this mutation to support tumorigenesis or metastasis (Robichaud et al., 2018; Wendel et al., 2007). Altogether, these results provide a mechanistic link between translational regulation and oncogene-driven alterations in myeloid cell fate, laying the groundwork for future studies aimed at dissecting the downstream molecular consequences of modulating this pathway.

Based on the identification of the Eif4ebp1-Eif4e1c axis as a mediator of oncogene silencing, and given that Mtor-mediated phosphorylation negatively regulates Eif4ebp1 activity, pharmacological inhibition of Mtor was expected to reinforce Eif4ebp1-mediated translational repression, thereby potentiating oncogene silencing in *mpx/eGFP-HRAS* larvae. This rationale is supported by previous studies identifying mTORC1 as a

critical regulator of cap-dependent mRNA translation through phosphorylation of 4E-BPs (Saxton & Sabatini, 2017). Consistent with this hypothesis, our findings demonstrate that rapamycin, an FDA/EMA-approved inhibitor of Mtor, markedly reduced HRAS-driven neutrophil expansion throughout larval development. Importantly, rapamycin treatment did not alter neutrophil numbers in *mpx:eGFP* larvae, indicating that Mtor inhibition specifically affects oncogene-driven leukemogenesis while largely preserving normal myelopoiesis and neutrophil homeostasis. This selective effect suggests a greater dependence of HRAS-transformed cells on Mtor-mediated translational control, uncovering a therapeutically actionable axis linking Mtor signaling to translational control of oncogene activity. Consistent with this interpretation, Christian et al. (2005) reported that low concentrations of rapamycin profoundly inhibited AML cell clonogenic capacity while sparing their normal counterparts.

In conclusion, our work identifies a previously unrecognized phenomenon of spontaneous “oncogene silencing” in zebrafish models of RAS-driven myeloid leukemia. Following an initial phase of oncogenic neutrophil expansion, HRAS-expressing cells progressively lose their oncogenic state and regress independently of external immune-mediated clearance, while NRAS-driven neutrophils display a similar transient expansion phenotype. Single-cell transcriptomic analyses further revealed marked heterogeneity within the neutrophil compartment, indicating that only a subset of neutrophils undergo full oncogenic transformation. Mechanistically, our findings demonstrate that this process is not dependent on Wnt/ β -catenin signaling, but is instead closely associated with translational control pathways, particularly the Mtor-Eif4ebp1-Eif4e1c axis. Within this regulatory network, Eif4ebp1 appears to function as a context-dependent suppressor of oncogenic neutrophil expansion, whereas Eif4e1c promotes both physiological and oncogenic myelopoiesis in a phosphorylation-dependent manner. Building on this mechanism, we identified Mtor as a pharmacological entry point to modulate oncogene silencing. Collectively, these findings establish translational control as a central regulator of neutrophil fate during oncogene silencing and position the zebrafish as a powerful *in vivo* system to uncover intrinsic mechanisms limiting oncogenic progression and to identify potential therapeutic vulnerabilities in RAS-driven malignancies.

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Author contributions

MLC and VM conceived the study; AJ-B, MF-L, IM-O and JC-S performed the research; AJ-B, MF-L, IM-O, JC-S, DG-M, FA-P, MLC and VM analyzed data; AJ-B wrote the original draft; VM edited the final version. All authors have read and agreed to the published version of the manuscript.

Competing interest statement

The authors declare no competing interests.

Data and biological materials availability

All generated materials/tools are available from the corresponding authors. The source data generated and/or analyzed during the development of this study will be provided as a Source Data file upon acceptance.

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Figure legends

Figure 1. Characterization of the Tg(*mpx:eGFP-HRAS_G12V*) transgenic line in larvae and adult fish. (A) a schematic representation of the transgenic zebrafish breeding strategies used to obtain the *mpx:eGFP;lyz:dsRED* line; b diagram of a zebrafish larva indicating the developing kidney (cyan), the CHT (pink), and the tail region (green box) used for neutrophil counting; c representative image of a 7-dpf *mpx:eGFP;lyz:dsRED* larva, shown in bright field, eGFP, dsRED, and merged channels. (B) a schematic representation of the transgenic zebrafish breeding strategies used to obtain the *mpx/eGFP-HRAS;lyz:dsRED* line; b diagram of a zebrafish larva indicating the developing kidney (cyan), the CHT (pink), and the tail region (red box) used for neutrophil counting; c representative image of a 7-dpf *mpx/eGFP-HRAS;lyz:dsRED* larva, shown in bright field, eGFP, dsRED, and merged channels. Schematic representations (Aa, Ab, Ba, Bb) were created with *BioRender.com*. Scale bars (Ac, Bc): 400 μ m. (C) Quantification of eGFP⁺ neutrophils in the tail region of 2- to 25-dpf *mpx/eGFP-HRAS;lyz:dsRED* larvae compared to *mpx:eGFP* controls. Each dot represents the neutrophil count of a single larva. The sample size (n), indicated below each column, corresponds to the number of larvae analyzed. (D) Representative images of the tail region of 15- and 25-dpf *mpx/eGFP-HRAS;lyz:dsRED* larvae. Scale bars: 400 μ m. (E) Quantification of eGFP⁺ and dsRED⁺ neutrophils within the tail region of 15-, 20-, and 25-dpf *mpx/eGFP-HRAS;lyz:dsRED* larvae. Each dot represents the neutrophil count of a single larva. The sample size (n), indicated below each column, corresponds to the number of larvae analyzed. (F) Representative flow cytometry dot plot of WKM cells from 3-mpf *mpx:eGFP* and *mpx/eGFP-HRAS* fish, showing defined hematopoietic populations: myeloid (orange), lymphoid (yellow), erythroid (blue), and precursor (pink) cells. eGFP⁺ neutrophils from *mpx:eGFP* WKM are shown in green. (G) Quantification of myeloid (orange), lymphoid (yellow), erythroid (blue), and precursor (pink) cells as a percentage of total WKM cells in 12 3-mpf *mpx:eGFP* controls (green) and *mpx/eGFP-HRAS* (red) fish (n = 12 per group). Each dot represents percentage of the indicated cell population in the WKM of an individual fish. Bars indicate mean $\pm$ SEM. dpf, days post fertilization; mpf, months post fertilization. ns, not significant; **p* < 0.05; *****p* < 0.0001, according to Kruskal–Wallis test followed by uncorrected Dunn’s test (C), or ordinary one-way ANOVA followed by uncorrected Fisher’s LSD test (E) or Šídák's multiple comparisons test (G).

Figure 2. Single-cell RNA-seq analysis of neutrophils in *mpx/eGFP-HRAS;lyz:dsRED* larvae at 8 dpf. (A) UMAP representation of all cells in the dataset, clustered into 24 transcriptionally distinct populations based on gene expression profiles. Each cluster is shown in a different color. (B) Expression of *mpx* (left) and *lyz* (right) projected onto the UMAP of all cells. Gene expression levels are shown as a color gradient from gray (low/no expression) to purple (high expression). While cells expression *mpx* and *lyz* are scattered throughout the map, the highest and most concentrated expression is observed in cluster 18, consistent with its neutrophil identity. (C) Reclustering of the neutrophil population (cluster 18). Left: UMAP showing only the cells from cluster 18 in salmon, previously identified as neutrophils (see A-B). Right: UMAP of the same neutrophil cells after reclustering, revealing six transcriptionally distinct subclusters (0-5), each represented by a different color. The arrow indicates the analytical step of reclustering. (D) Feature plots showing the expression of the canonical neutrophil markers *mpx* (left) and *lyz* (right) projected onto the UMAP of neutrophil subclusters. Gene expression levels are represented by a color gradient from gray (low/no expression) to purple (high expression). Cell expressing *mpx* and *lyz* are distributed across all six subclusters, with variable expression levels within each. (E) *HRAS* expression across neutrophil subclusters. Left: Binary feature plot showing individual *HRAS*-positive (salmon) and *HRAS*-negative (blue) cells projected onto the UMAP of the six neutrophil subclusters. Right: Stacked bar graph representing the proportion of *HRAS*-positive (salmon) and *HRAS*-negative (blue) cells within each subcluster. Subclusters 0, 1, 2, and 4 contain a higher proportion of *HRAS*-positive cells, while subclusters 3 and 5 are predominantly *HRAS*-negative. (F) Violin plots showing the expression of selected genes across neutrophil subclusters. Left: *ctnnb1* expression is enriched in subclusters 1 and 4. Right: *ef4ebp1* expression is selectively enriched in subcluster 1. The y-axis indicates normalized expression levels, and each point represents a single cell. The width of the violin indicates the density of cells at a given expression level.

Figure 3. Effect of Wnt/ β -catenin pathway pharmacological inhibition on oncogene silencing in *mpx/eGFP-HRAS* zebrafish larvae. (A) Schematic of the Wnt/ β -catenin pathway including the XAV-939 target site. Left: activated pathway allowing β -catenin-mediated transcription. Right: inhibited pathway in the presence of XAV-939, which stabilizes the Axin/CK1 α /APC/GSK-3 β complex, leading to β -catenin phosphorylation and subsequent ubiquitin-mediated proteasomal degradation, thereby preventing nuclear

translocation and transcriptional activation. (B) Schematic overview of the experimental design (created with *BioRender.com*). *mpx/eGFP-HRAS* embryos were raised without treatment until 3 dpf, then exposed to either 1% DMSO or 1 μ M XAV-939 from 3 to 24 dpf. Larvae were monitored and imaged at selected time points, and eGFP⁺ neutrophils were quantified in the tail region. (C) Representative images of the tail region of *mpx/eGFP-HRAS* larvae at 10 and 12 dpf (7 and 9 days post-treatment, dpt, respectively), treated with DMSO (top) or 1 μ M XAV-939 (bottom), showing eGFP⁺ neutrophils. Scale bars: 400 μ m. (D) Quantification of eGFP⁺ neutrophils in the tail region from 5 to 24 dpf in DMSO- and XAV-939-treated larvae. No significant differences were observed at any time point. Each dot represents the neutrophil count of a single larva. The total number of larvae analyzed (n) is indicated below each column; bars represent the mean $\pm$ SEM. dpf, days post fertilization. ns, not significant, according to Kruskal–Wallis test followed by uncorrected Dunn’s test.

Figure 4. Effect of CRISPR/Cas9-mediated disruption of *eif4ebp1* on neutrophil counts in zebrafish larvae.

(A) Schematic representation of regulation of cap-dependent mRNA translation by ERK–MNK and mTORC1–4E-BP signaling. Phosphorylation of eIF4E by MNK downstream of ERK promotes assembly of the eIF4F translation initiation complex (eIF4E, eIF4G, and eIF4A) and activation of cap-dependent mRNA translation. In parallel, mTORC1-mediated phosphorylation of 4E-BPs prevents their binding to eIF4E, allowing eIF4F complex formation. In contrast, unphosphorylated 4E-BPs bind to eIF4E and prevent eIF4F complex formation, thereby inhibiting translation initiation. 4E-BPs include EIF4EBP1; eIF4E proteins include eIF4E1C. (B) Representative fluorescence images of the tail region of 12-dpf zebrafish larvae injected at the one-cell stage with either control or *eif4ebp1*-targeting crRNAs. In *mpx/eGFP-HRAS;lyz:dsRED* larvae (top panels), merged fluorescence and individual eGFP (green) and dsRED (red) channels are shown. In *lyz:dsRED* larvae (bottom panels), the individual dsRED (red) channel is shown. Scale bars: 400 μ m. (C–E) Quantification of neutrophils in *mpx/eGFP-HRAS;lyz:dsRED* larvae (eGFP⁺, C; dsRED⁺, D) and *lyz:dsRED* larvae (dsRED⁺, E) from 5 to 12 dpf. Each dot represents the neutrophil count of a single larva. The total number of larvae analyzed (n) is indicated below each column; bars represent the mean $\pm$ SEM. dpf, days post fertilization. ns, not significant, * p < 0.05; ** p < 0.01; **** p < 0.0001, according to Kruskal–Wallis test followed by uncorrected Dunn’s test.

Figure 5. Comparative effect of CRISPR/Cas9-mediated disruption of *EIF4EBP1* and *EIF4E1C* on neutrophil counts in zebrafish larvae. (A) Schematic overview of the experimental design (created with *BioRender.com*). *mpx:eGFP-HRAS* and *mpx:eGFP* embryos were injected at the one-cell stage with a CRISPR/Cas9 microinjection mixture containing either control (non-targeting) crRNA, *EIF4EBP1*-targeting crRNA, or two *EIF4E1C*-targeting crRNAs (crRNA1 and crRNA2), and raised until 7 dpf. At this stage, half of the progeny were processed for Sanger sequencing and ICE analysis to validate gene editing efficiency, while the other half were imaged to quantify eGFP⁺ neutrophils in the tail region. (B, C) Representative fluorescence images (B) and quantification (C) of eGFP⁺ neutrophils in the tail region of 7-dpf *mpx:eGFP-HRAS* larvae injected with the crRNAs described in panel A. (D, E) Representative fluorescence images (D) and quantification (E) of eGFP⁺ neutrophils in 7-dpf *mpx:eGFP* larvae injected with the crRNAs described in panel A. Scale bars (B, D): 400 μ m. In (C, E) each dot represents the neutrophil count of a single larva. The total number of larvae analyzed (n) is indicated below each column; bars represent the mean $\pm$ SEM. dpf, days post fertilization. ns: not significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, according to Kruskal–Wallis test followed by Dunn’s multiple comparisons test.

Figure 6. Comparative effects of WT and S205A *EIF4E1C* overexpression on neutrophil counts in zebrafish larvae. (A) Schematic overview of the experimental design (created with *BioRender.com*). *mpx:eGFP-HRAS* and *mpx:eGFP* embryos were injected at the one-cell stage with mRNAs encoding GFP (control), WT Eif4e1c, or Eif4e1c^{S205A}, and raised until 3 dpf. At this stage, half of the progeny were processed for RT-qPCR to validate microinjection efficiency, while the other half were imaged to quantify eGFP⁺ neutrophils in the tail region. (B, C) Representative fluorescence images (B) and quantification (C) of eGFP⁺ neutrophils in the tail region of 3-dpf *mpx:eGFP-HRAS* larvae injected with the mRNAs described in panel A. (D, E) Representative fluorescence images (D) and quantification (E) of eGFP⁺ neutrophils in the tail region of 3-dpf *mpx:eGFP* larvae injected with the mRNAs described in panel A. (F) Schematic overview of the experimental design (created with *BioRender.com*). *lyz:mVENUS-NRAS;mpx:eGFP* embryos were uninjected or injected at the one-cell stage with plasmids containing either the *lyz:eif4e1c* (WT) or *lyz:eif4e1c*^{S205A} construct. Larvae were raised and imaged at 5, 7, and 12 dpf for quantification of eGFP⁺ neutrophils in the tail region.

(G, H) Representative images of the tail region of 12-dpf *lyz:mVENUS-NRAS;mpx:eGFP* larvae (G) and quantification of eGFP⁺ neutrophils at 5, 7, and 12 dpf (H). Larvae were either uninjected or injected with plasmids described in panel F. Scale bars (B, D, G): 400 μ m. In (C, E, H) each dot represents the neutrophil count of a single larva. The total number of larvae analyzed (n) is indicated below each column; bars represent the mean $\pm$ SEM. dpf, days post fertilization. ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, according to ordinary one-way ANOVA followed by Tukey's multiple comparisons test (C), Kruskal–Wallis test followed by Dunn's multiple comparisons test (E), or Kruskal–Wallis test followed by uncorrected Dunn's test (H).

Figure 7. Effect of Mtor pharmacological inhibition on oncogene silencing in *mpx/eGFP-HRAS* zebrafish larvae. (A) Left: Schematic overview of the RAS downstream signaling pathways highlighting the mTORC1/4E-BP axis (created with *BioRender.com*). Right: Enlarged view of the mTORC1/4E-BP axis illustrating the mechanism of rapamycin-mediated inhibition of mTOR signaling. Right, top: In the absence of rapamycin, active mTORC1 phosphorylates 4E-BPs, preventing their interaction with eIF4E and allowing assembly of the eIF4F translation initiation complex (eIF4E, eIF4G, and eIF4A), thereby promoting cap-dependent mRNA translation. Right, bottom: Rapamycin binds FKBP12, and the resulting FKBP12–rapamycin complex binds to mTOR within the mTORC1 complex, inhibiting its kinase activity and thereby preventing phosphorylation of 4E-BPs. Hypophosphorylated 4E-BPs bind eIF4E, blocking eIF4F complex assembly and repressing cap-dependent mRNA translation. The three-dimensional representation of the mTORC1 complex was adapted from Saxton & Sabatini (2017). (B) Schematic overview of the experimental design (created with *BioRender.com*). *mpx/eGFP-HRAS* and *mpx:eGFP* embryos were raised without treatment until 2 dpf, then exposed to either 0.2% DMSO, or 1 or 10 μ M rapamycin from 2 to 7 dpf. Larvae were monitored and imaged at selected time points, and eGFP⁺ neutrophils were quantified in the tail region. (C) Representative images of the tail region of *mpx/eGFP-HRAS* larvae at 7 dpf (5 days post-treatment, dpt), treated with DMSO (top), 1 μ M rapamycin (middle), or 10 μ M rapamycin (bottom), showing eGFP⁺ neutrophils. Scale bars: 400 μ m. (D) Time-course analysis of eGFP⁺ neutrophil numbers in the tail region of *mpx:eGFP-HRAS* larvae treated with DMSO, 1 μ M rapamycin, or 10 μ M rapamycin. Each dot represents the mean neutrophil count (10 larvae per group). (E, G) Representative images of the tail region of *mpx/eGFP-HRAS* (E) and *mpx:eGFP* (G)

larvae at 5 dpf (3 days post-treatment, dpt), treated with DMSO (top) or 10 μ M rapamycin (bottom), showing eGFP⁺ neutrophils. Scale bars: 400 μ m. (F, H) Quantification of eGFP⁺ neutrophils in the tail region of DMSO- and rapamycin-treated *mpx:eGFP-HRAS* (F) and *mpx:eGFP* (H) larvae at 3, 5, and 7 dpf. Each dot represents the neutrophil count of a single larva. The total number of larvae analyzed (n) is indicated below each column. (D, F, H) bars represent the mean $\pm$ SEM. dpf, days post fertilization. ns: not significant; * p < 0.05; *** p < 0.001; **** p < 0.0001, according to two-way ANOVA followed by Tukey's multiple comparisons test (D), ordinary one-way ANOVA followed by uncorrected Fisher's LSD test (F), or Kruskal–Wallis test followed by uncorrected Dunn's test (H).

Supplementary Figure S1. Characterization of the Tg(*lyz:mVENUS-NRAS_Q61L*)

transgenic line in larvae and adult fish. (A) a schematic representation of the transgenic zebrafish breeding strategy used to obtain the *lyz:mVENUS-NRAS;mpx:eGFP;lyz:dsRED* line; b diagram of a zebrafish larva indicating the developing kidney (cyan), the CHT (pink), and the tail region (blue box) used for neutrophil counting. Both schematics were created with *BioRender.com*; c representative image of a 7-dpf *lyz:mVENUS-NRAS;mpx:eGFP;lyz:dsRED* larva, shown in bright field, eGFP, dsRED, and merged channels. Scale bar: 400 μ m. (B) Quantification of eGFP⁺ neutrophils in the tail region of 3- to 14-dpf *lyz:mVENUS-NRAS;mpx:eGFP* larvae compared to *mpx:eGFP* controls. Each dot represents the neutrophil count of a single larva. The total number of larvae analyzed (n) is indicated below each column. (C) Representative images of adult *lyz:mVENUS-NRAS* fish at 23 mpf, ranked by physical condition from deteriorated (red) to healthy (green). Fish are numbered 1-6 for reference. Scale bar: 1 cm. (D) Representative flow cytometry dot plot of WKM cells from 23-mpf *mpx:eGFP* and *lyz:mVENUS-NRAS* fish, showing defined hematopoietic populations: myeloid (orange), lymphoid (fuchsia), erythroid (cyan), and precursor (blue) cells. (E) Quantification of myeloid cells as a percentage of total WKM cells in 23-mpf *mpx:eGFP* controls (gray) and *lyz:mVENUS-NRAS* (colored by physical condition) fish. (F) Quantification of *mVENUS-NRAS* expression by RT-qPCR in WKM samples from 23-mpf *lyz:mVENUS-NRAS* fish (color-coded by physical condition; fish 2–5) and pooled 6-dpf *lyz:mVENUS-NRAS* larvae (positive control, violet), normalized to *rps11* and expressed as fold change relative to the larval sample (set as 1). Each dot represents a technical replicate; bars indicate the mean $\pm$ SD. (G) Quantification of myeloid cells as a percentage of total WKM

cells in 10 mpf WT controls (gray) and 9 mpf *lyz:mVENUS-NRAS* fish (blue). In (E, G), each dot represents the WKM sample from an individual fish. In (B, E, G), bars indicate mean $\pm$ SEM. dpf, days post fertilization; mpf, months post fertilization. ns, not significant; **** $p < 0.0001$, according to Kruskal–Wallis test followed by uncorrected Dunn’s test (B), unpaired Student’s *t*-test (E, G), or ordinary one-way ANOVA followed by uncorrected Dunnett’s multiple comparisons test (F).

Supplementary Figure S2. Design and validation of *elf4ebp1* crRNA for CRISPR/Cas9-mediated gene editing in zebrafish. (A) Schematic representation of the zebrafish *elf4ebp1* genomic locus showing its four exons highlighted in color: exon 1 (magenta), exon 2 (blue), exon 3 (yellow), and exon 4 (cyan). The start codon located in exon 2 is indicated by a green triangle labeled “Met”, and the stop codon in exon 4 by a red square labeled “STOP”. The lower panel shows a magnified view of the genomic region encompassing exon 2, indicating the crRNA target site within exon 2 (orange) and the position of the validation primers flanking the Cas9 cleavage site (purple). Created with *Biorender.com*. (B) Workflow for verifying DNA conservation of the *elf4ebp1* target locus in adult zebrafish founder lines, including tissue sampling, DNA extraction, *elf4ebp1* locus amplification, PCR product size analysis and purification, and Sanger sequencing. (C) Magnified view of the Sanger sequencing chromatogram of the region encompassing the crRNA binding site sequence (shaded in orange), confirming its conservation in *mpx:eGFP*, *lyz:dsRED*, *mpx:GAL4* and *UAS:eGFP-HRAS* transgenic lines. (D) ICE analysis of editing outcomes in *elf4ebp1*-crRNA-injected larvae. The summary includes the crRNA target and PAM sequences, editing efficiency (indel percentage = 78%), model fit ($R^2 = 0.93$), knockout score (69), and the relative contribution of major sequence variants. A knockout score of 69 indicates that approximately 69% of the edited alleles are predicted to result in gene disruption.

Supplementary Figure S3. Validation of CRISPR/Cas9-mediated *elf4ebp1* and *elf4elc* editing efficiency and *elf4elc* overexpression. (A) Efficiency of *elf4ebp1* and *elf4elc* editing in F0 zebrafish larvae, assessed by ICE analysis from Sanger sequencing traces. Table shows ICE indel percentage, ICE model fit (R^2), and ICE knockout score for two independent experiments. (B) Representative RT-qPCR showing *elf4elc* transcript levels (normalized to *rps11*) in pooled 3-dpf larvae from WT and mutant *elf4elc* mRNA-injected groups (*mpx:eGFP-HRAS* background), presented as fold change

1491 relative to the GFP-injected control group (set as 1). Each dot represents a biological
1492 replicate; bars represent the mean $\pm$ SD. ns: not significant; $**p < 0.01$, according to
1493 ordinary one-way ANOVA followed by Tukey's multiple comparisons test.
1494

Figure 1

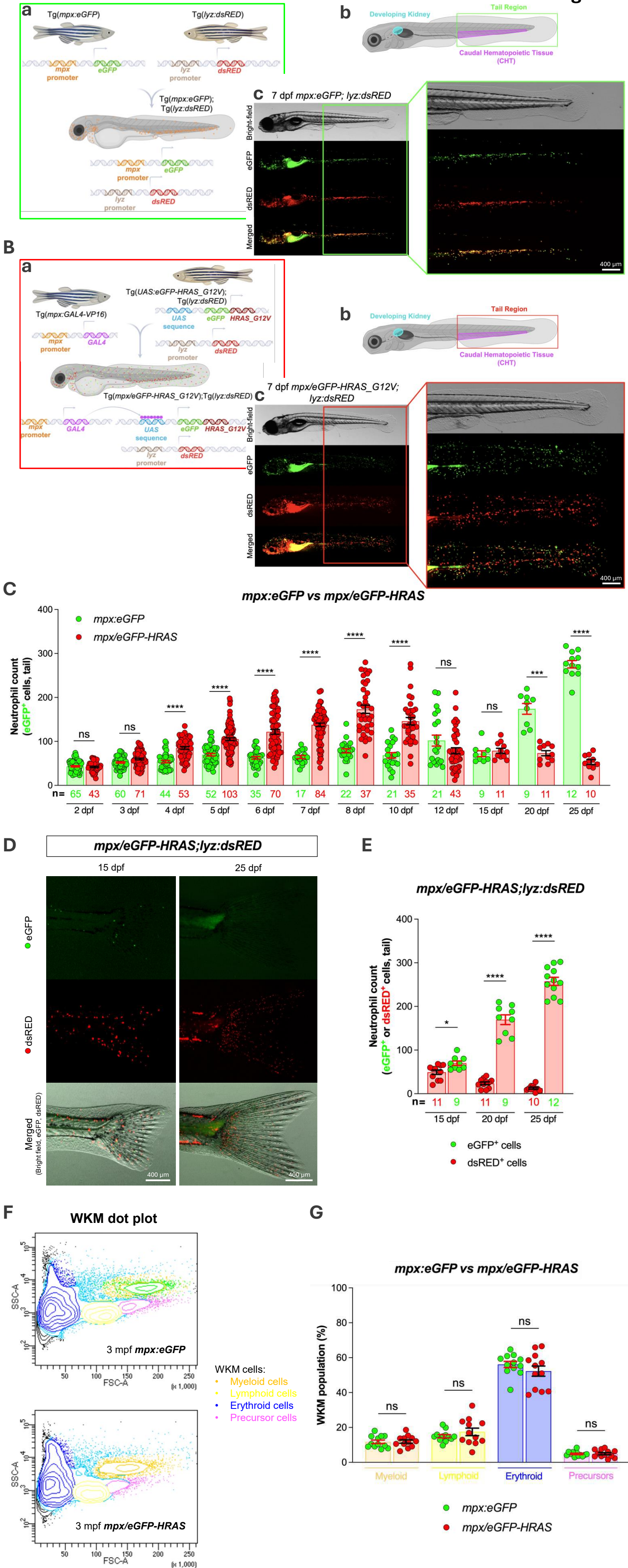
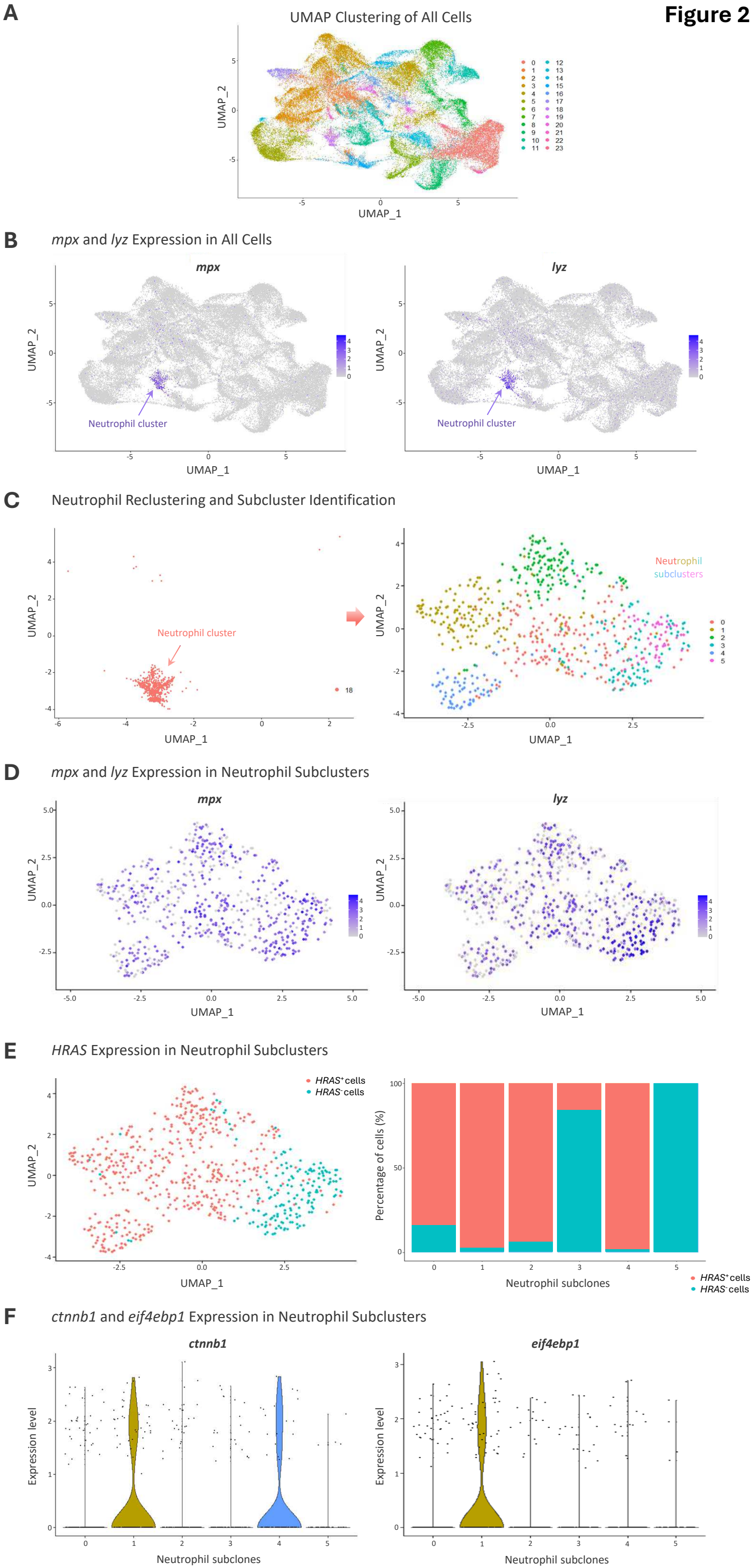


Figure 2



A

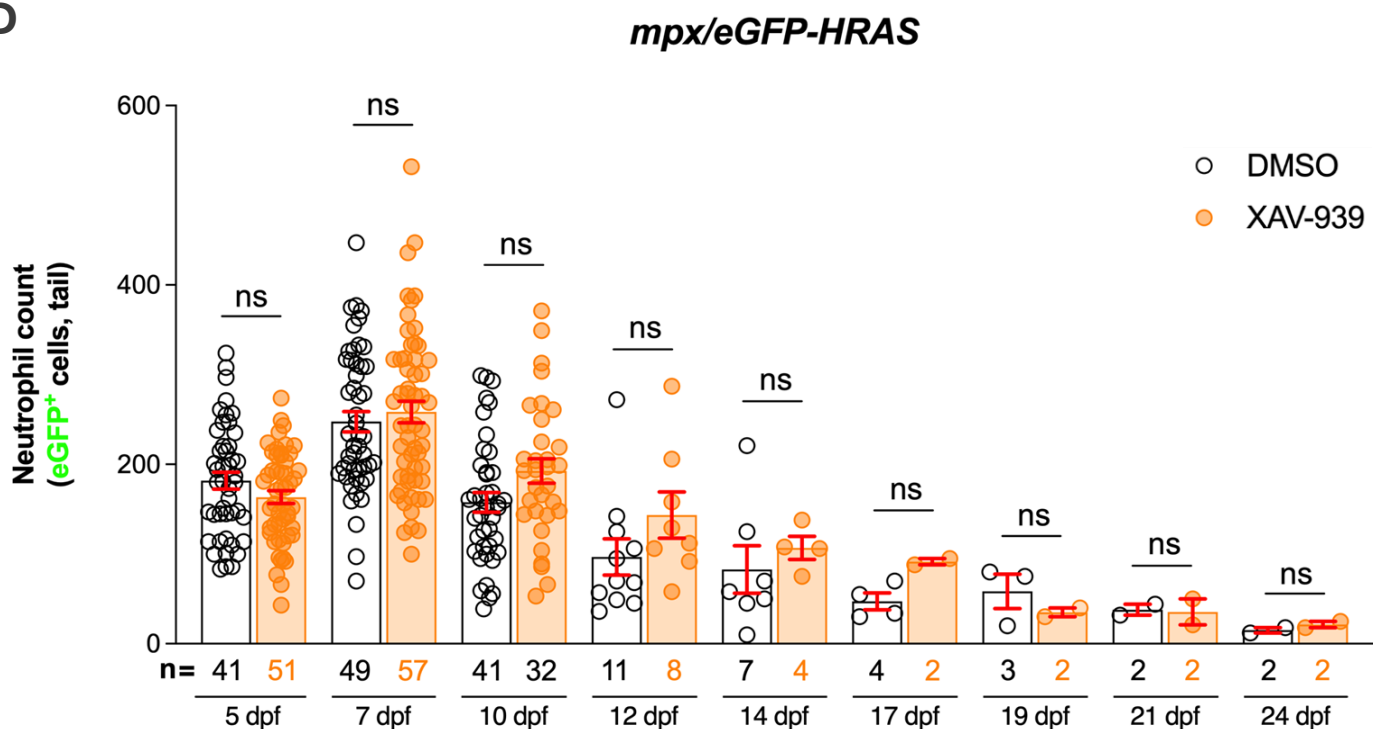


Figure 4

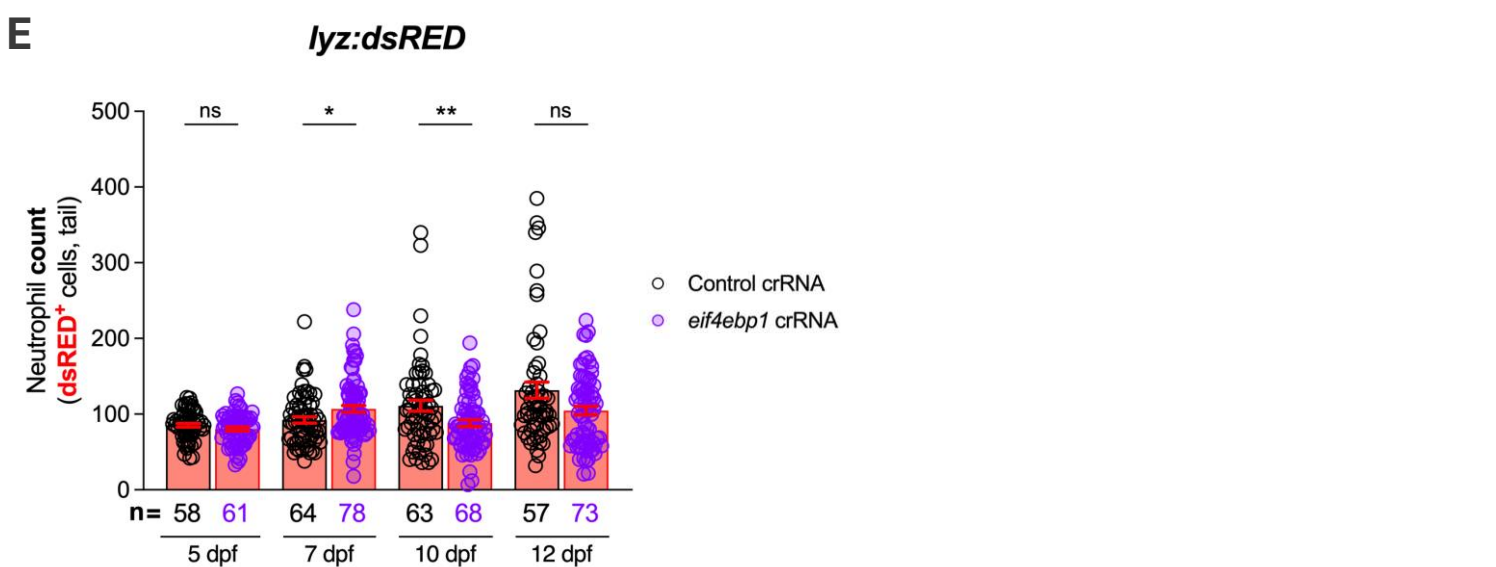
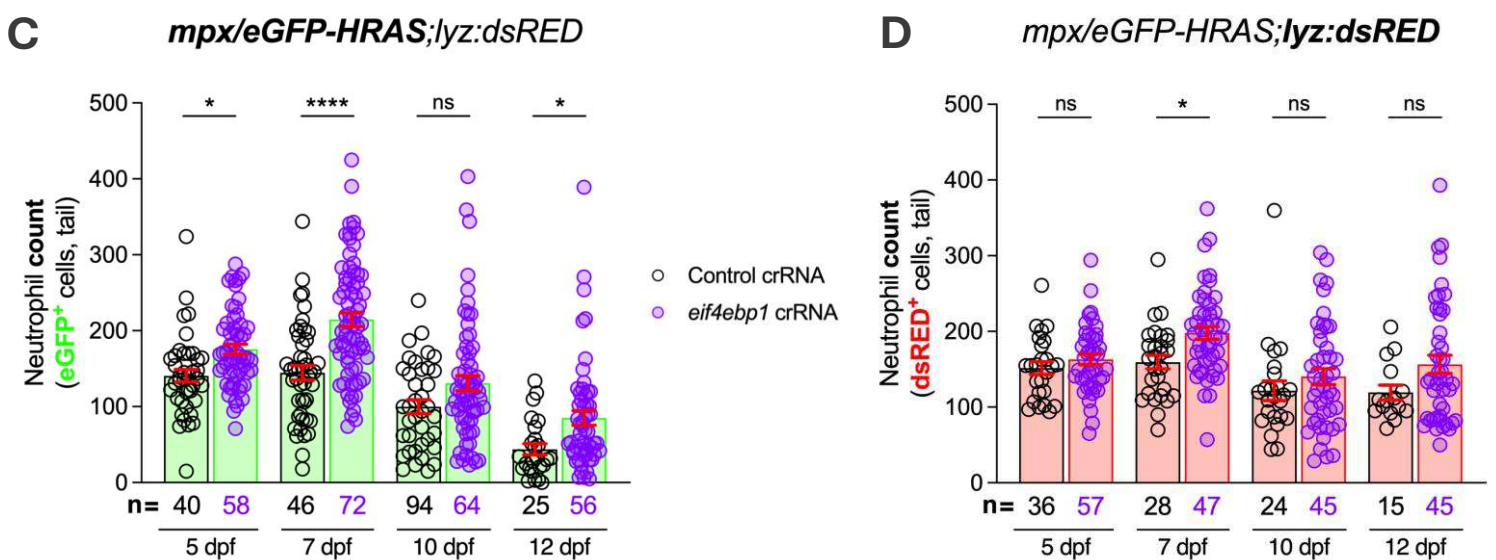
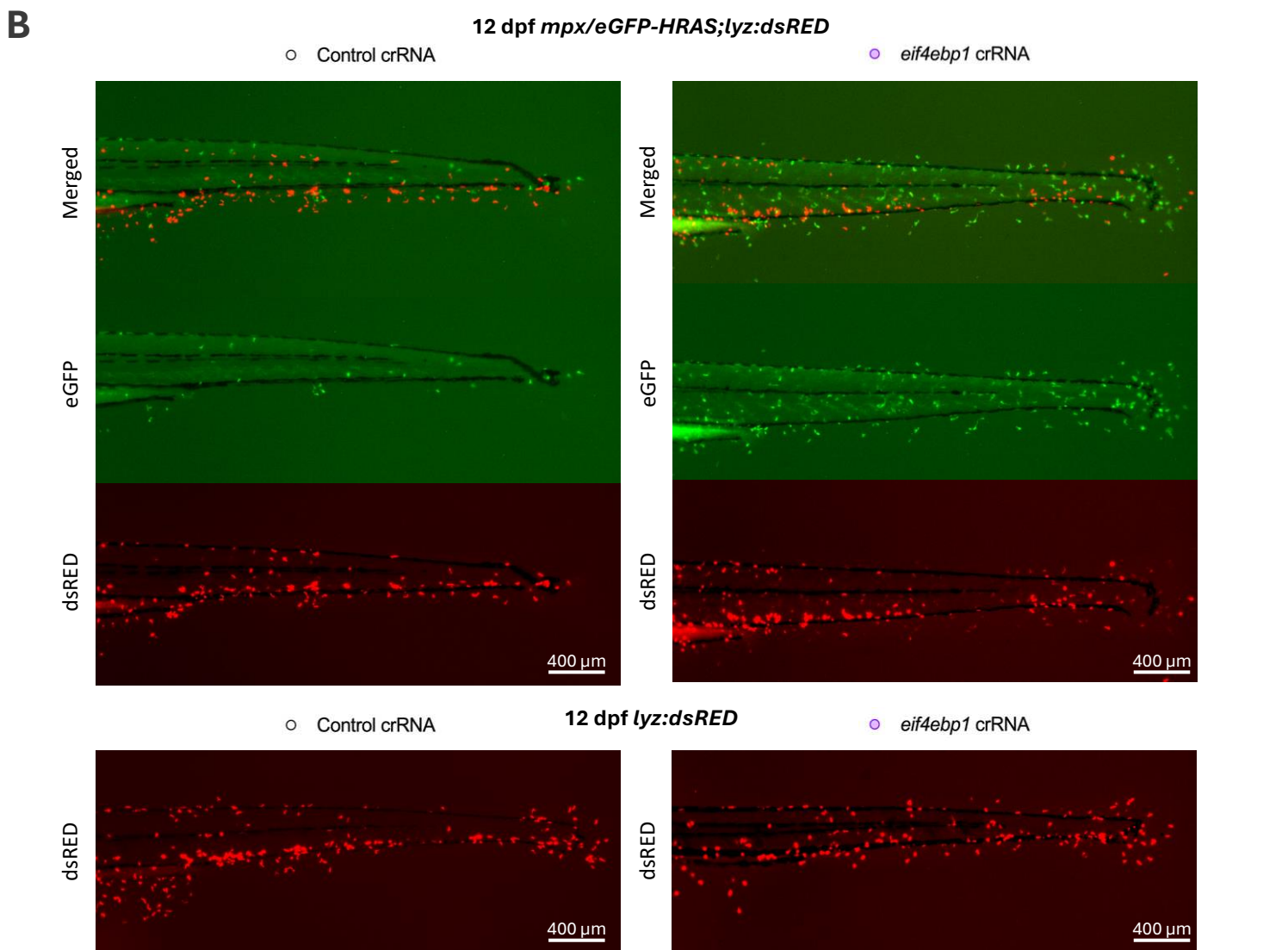
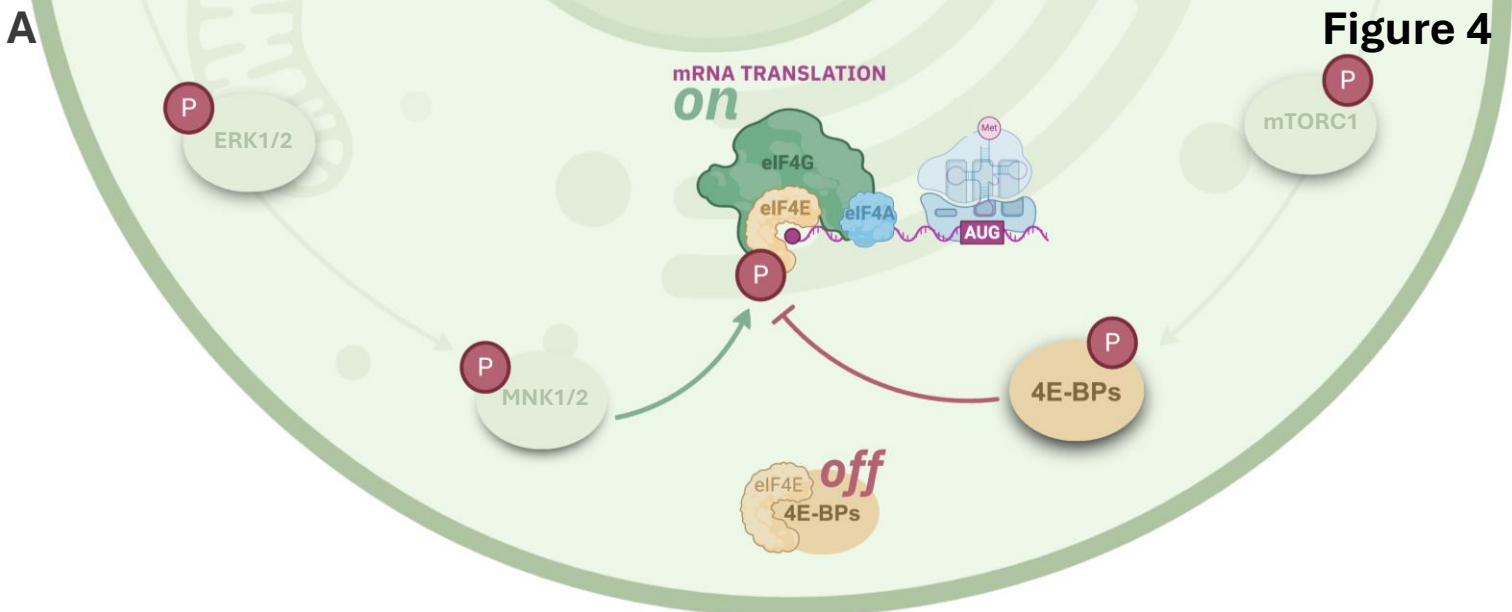
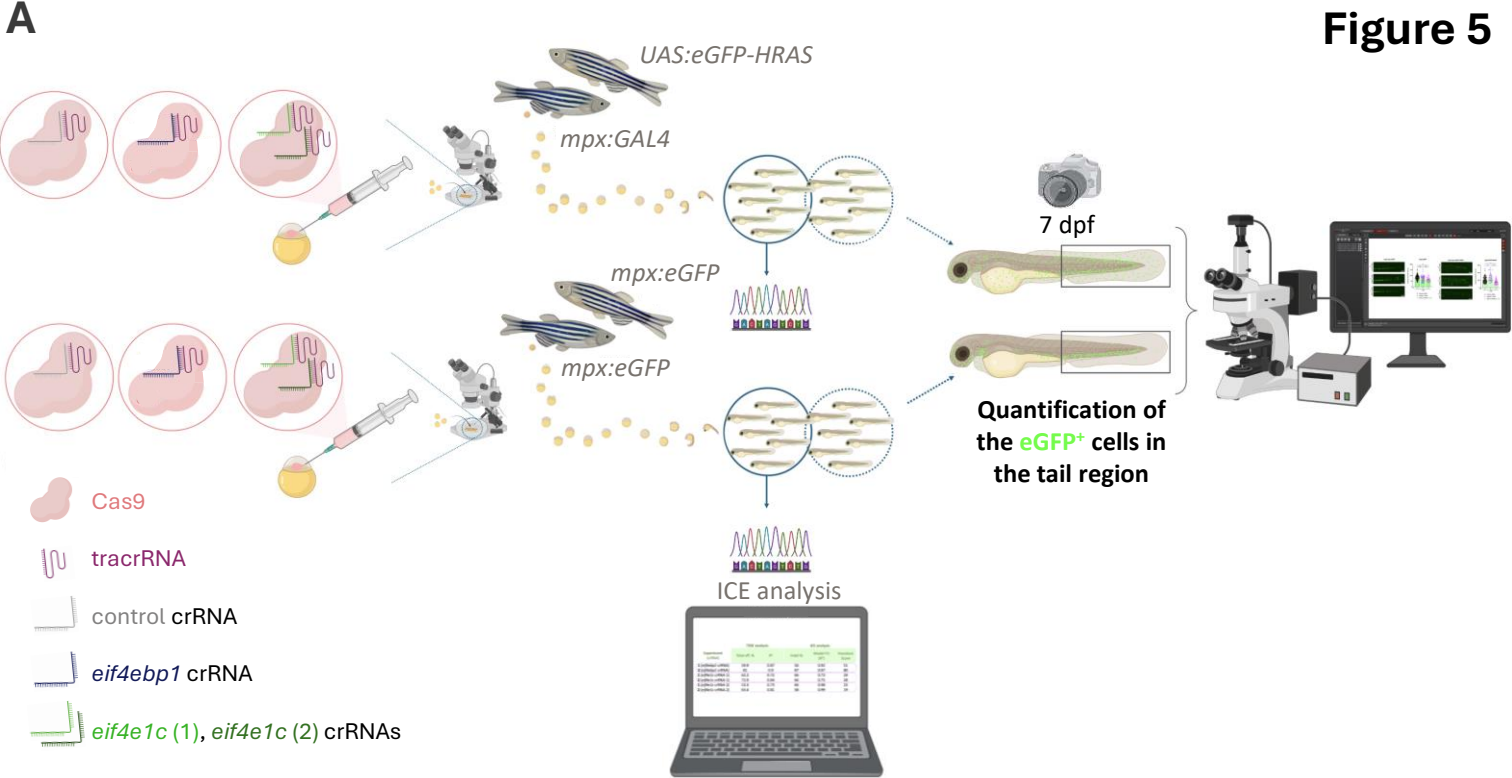
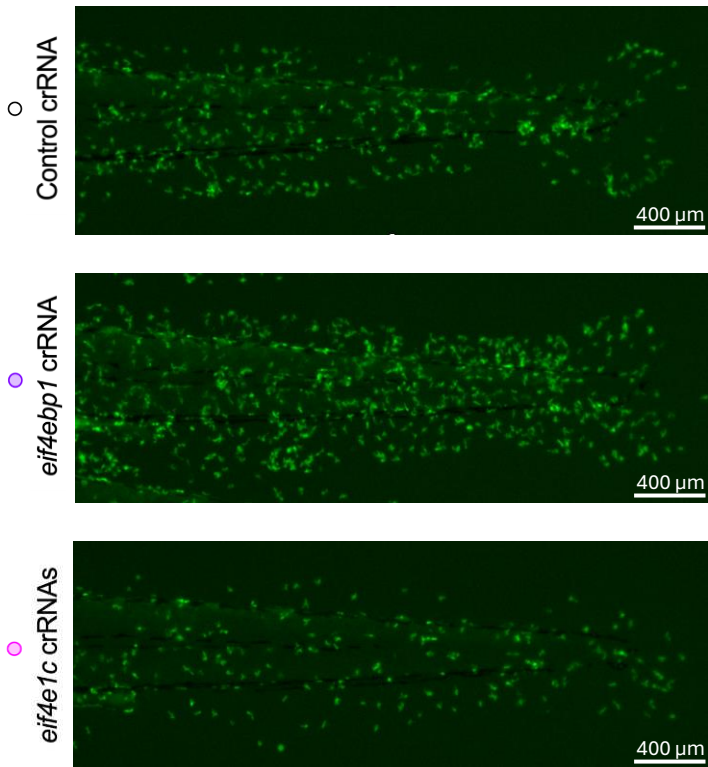


Figure 5



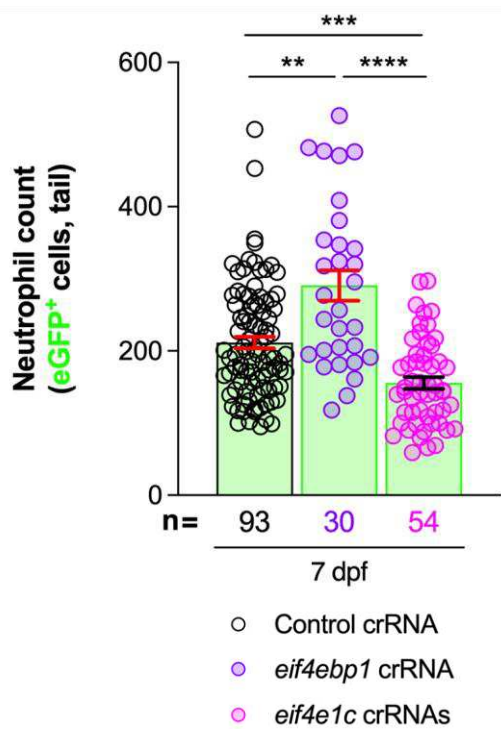
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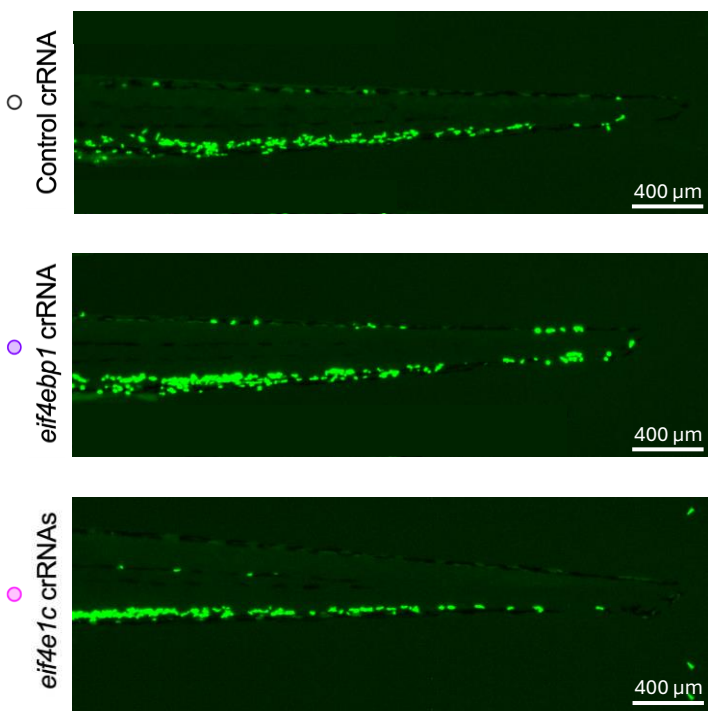
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mpx/eGFP-HRAS



D

7 dpf *mpx*:eGFP



E

mpx:eGFP

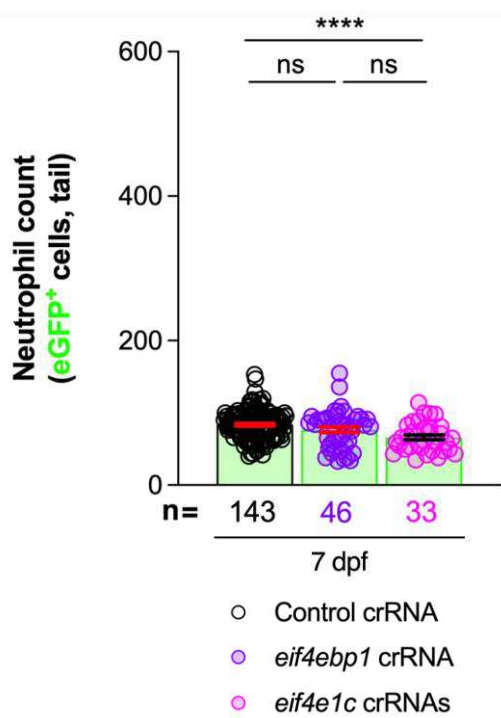
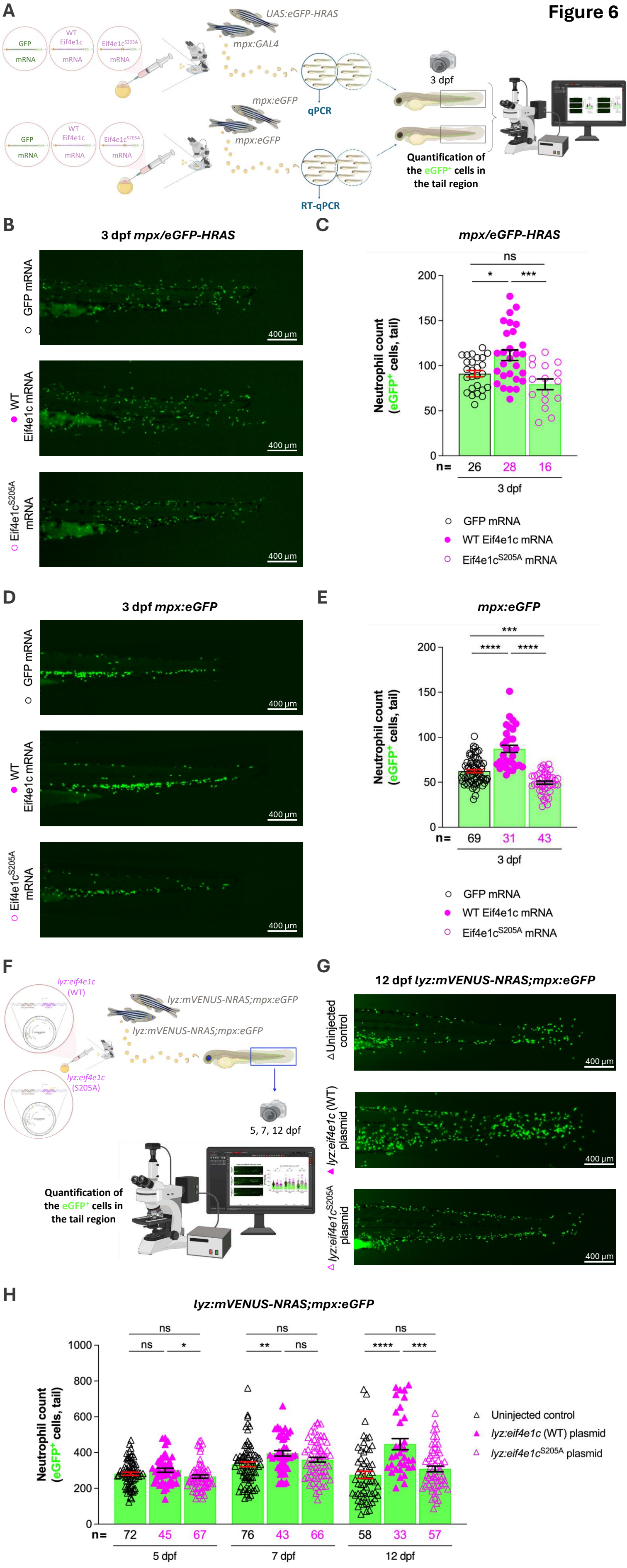


Figure 6



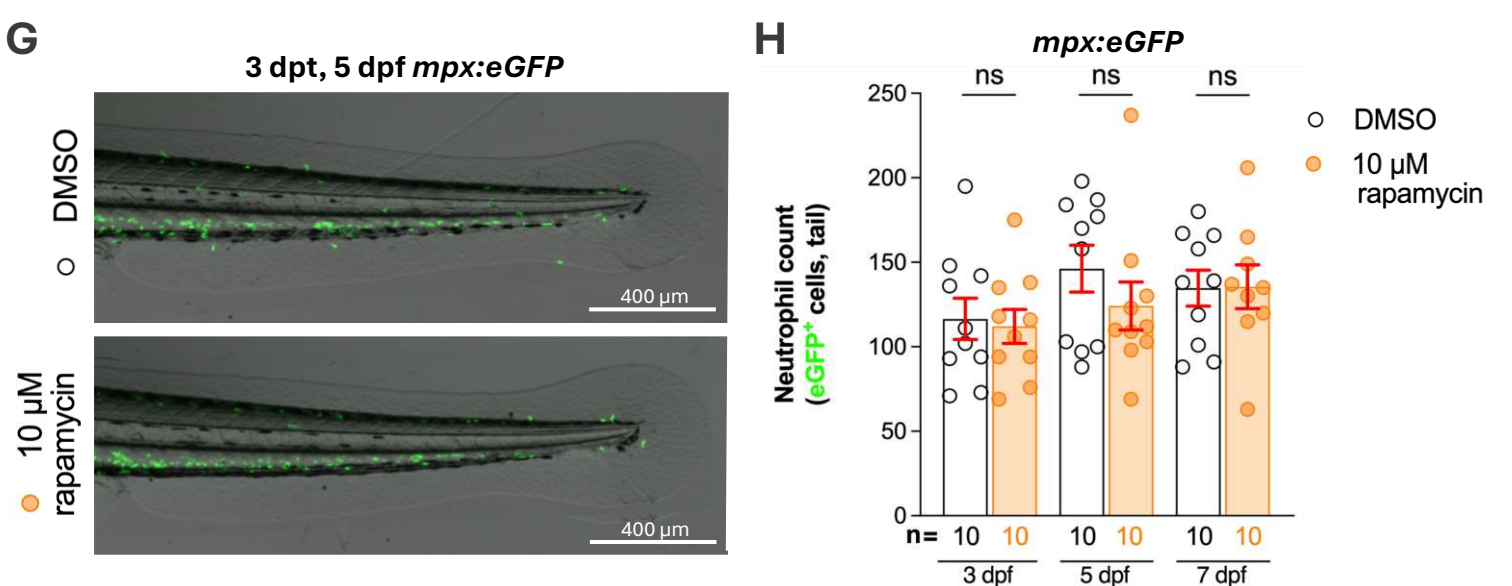
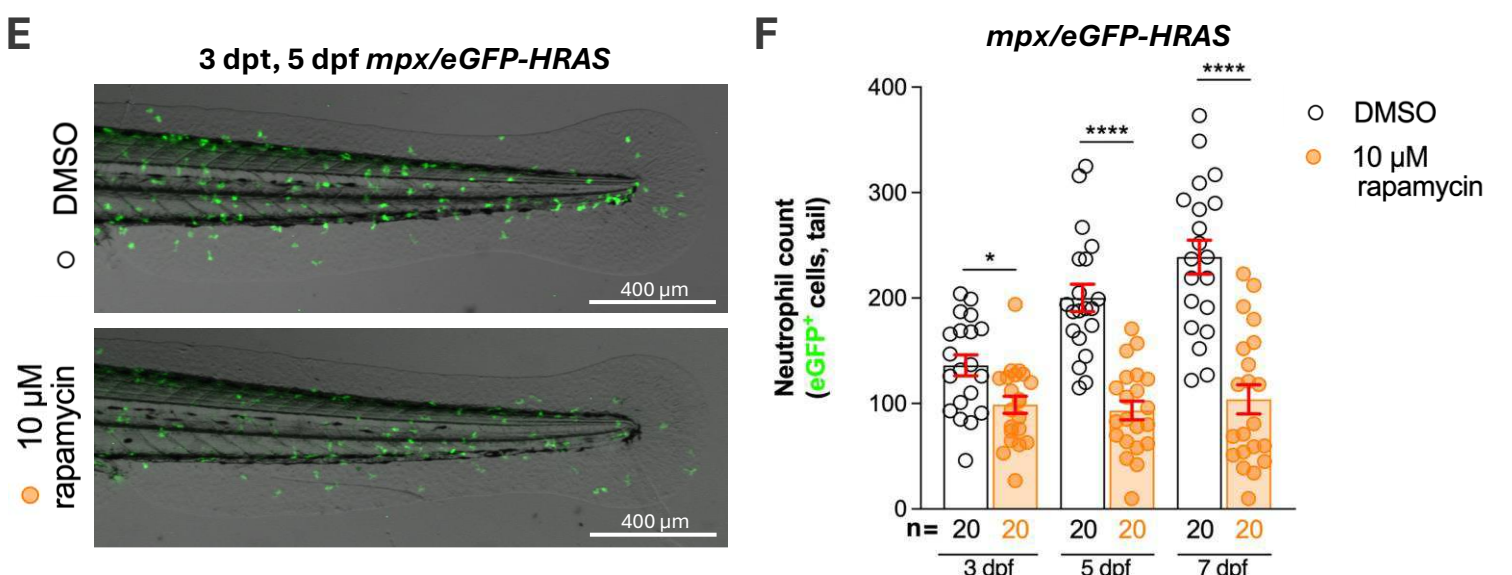
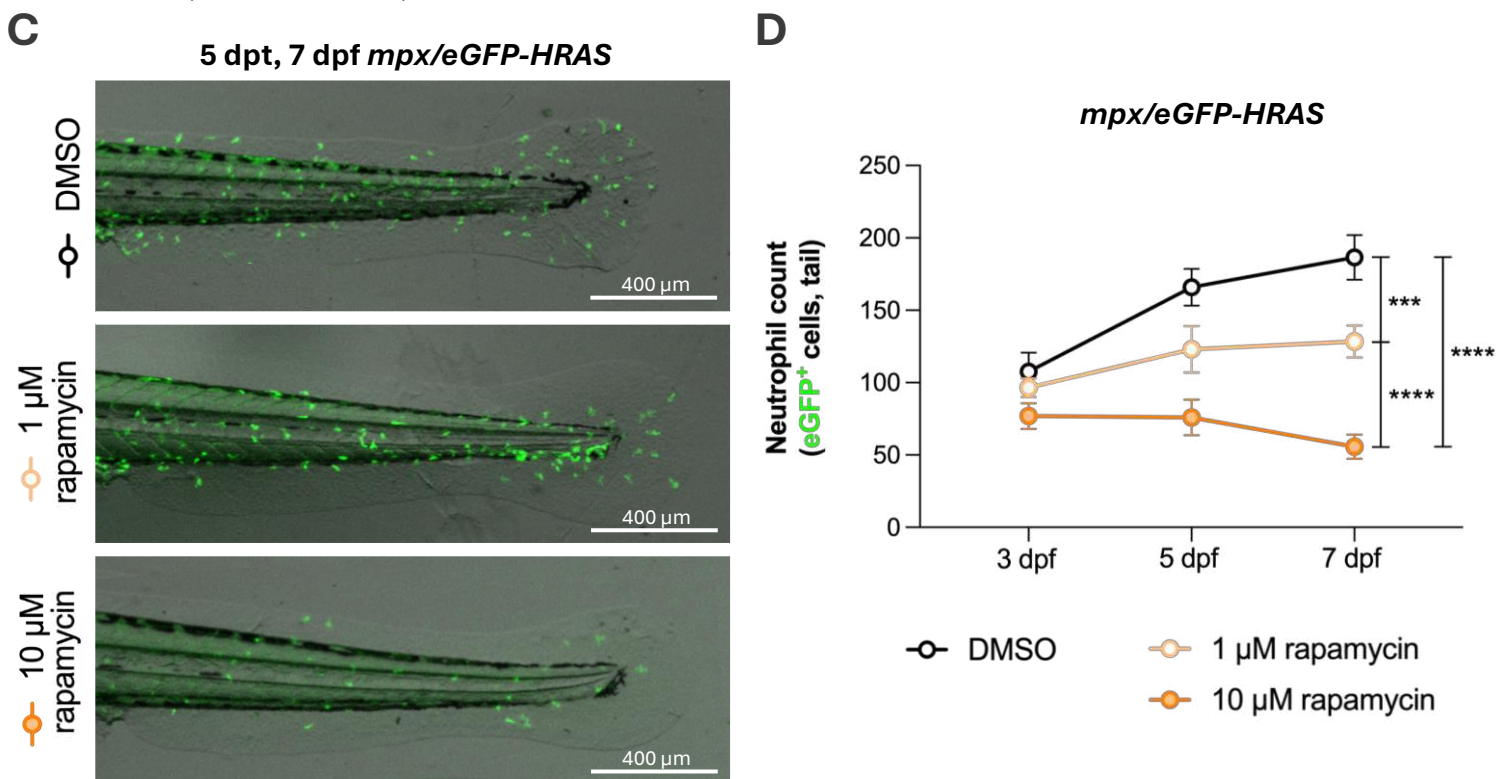
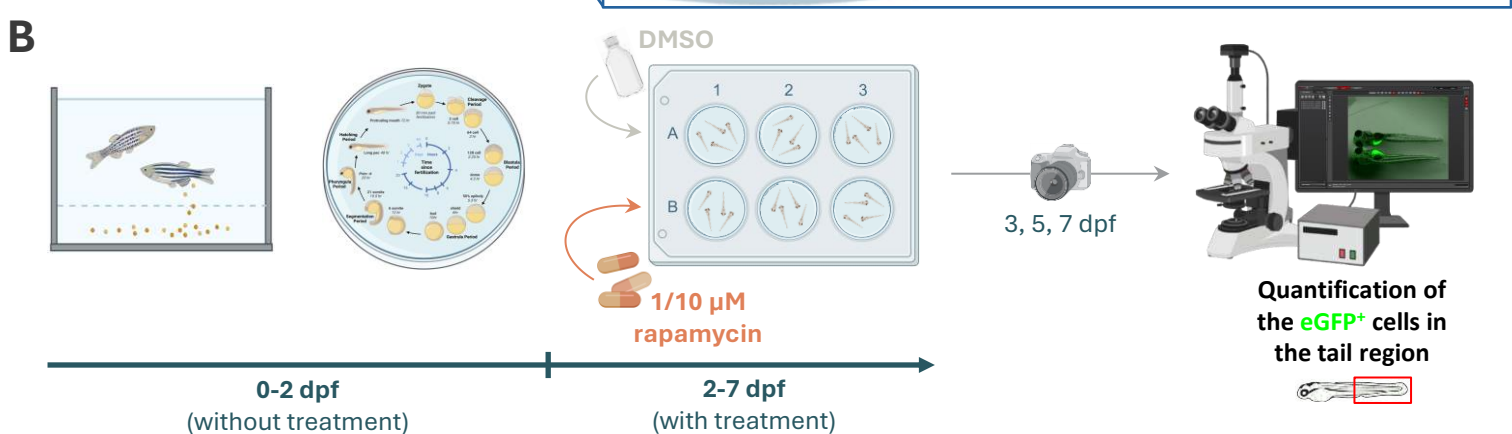
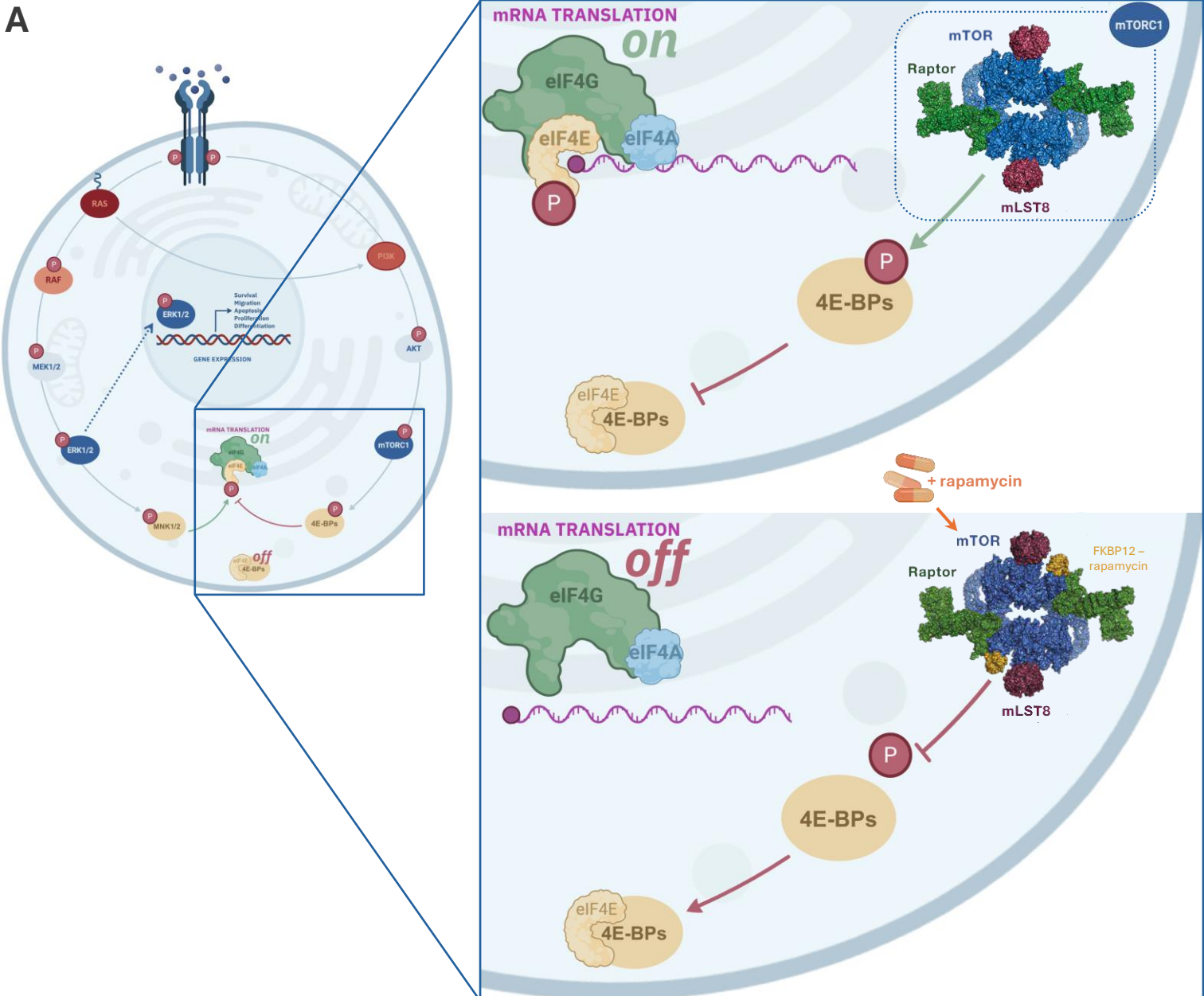
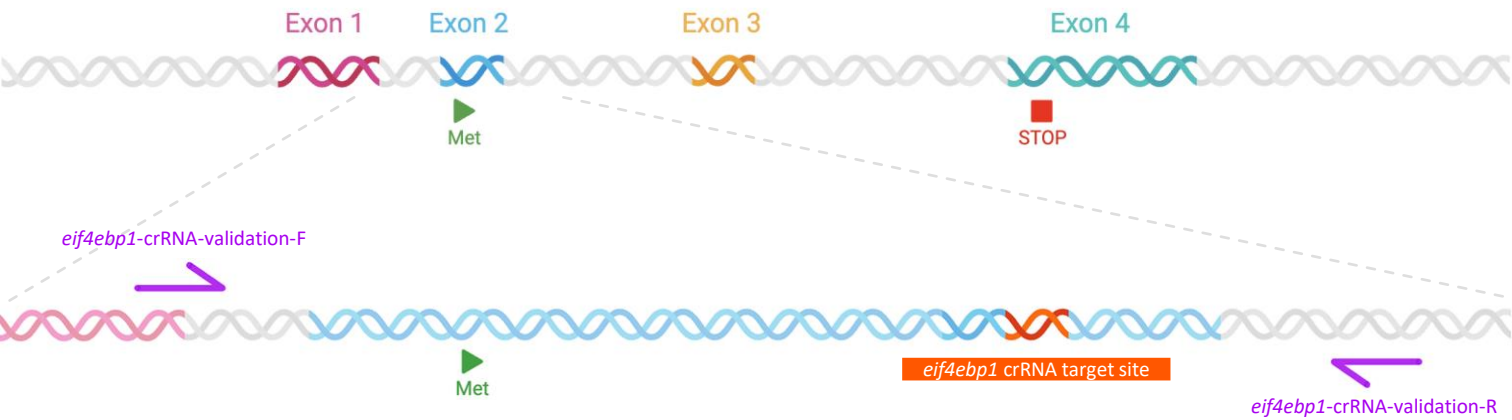
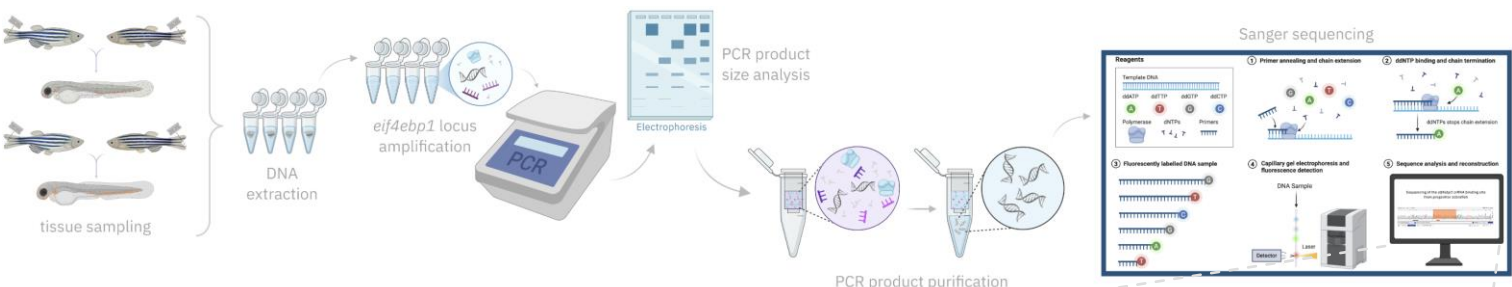


Figure 7

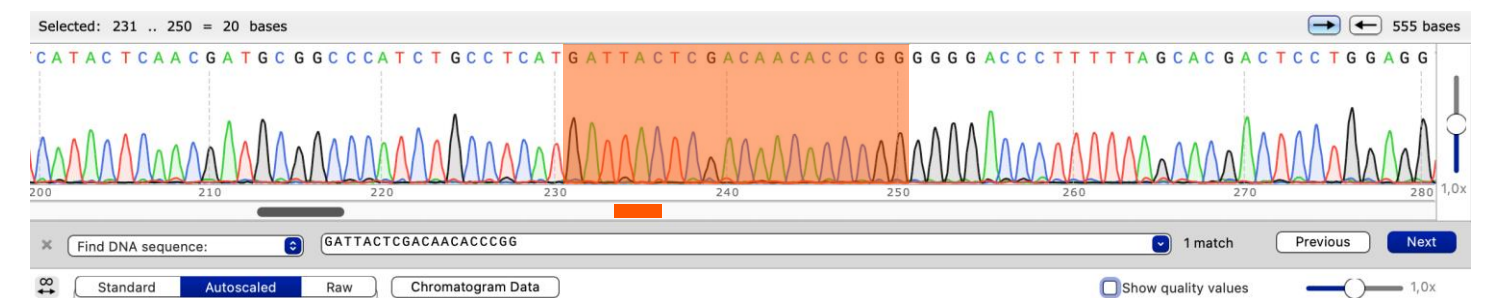




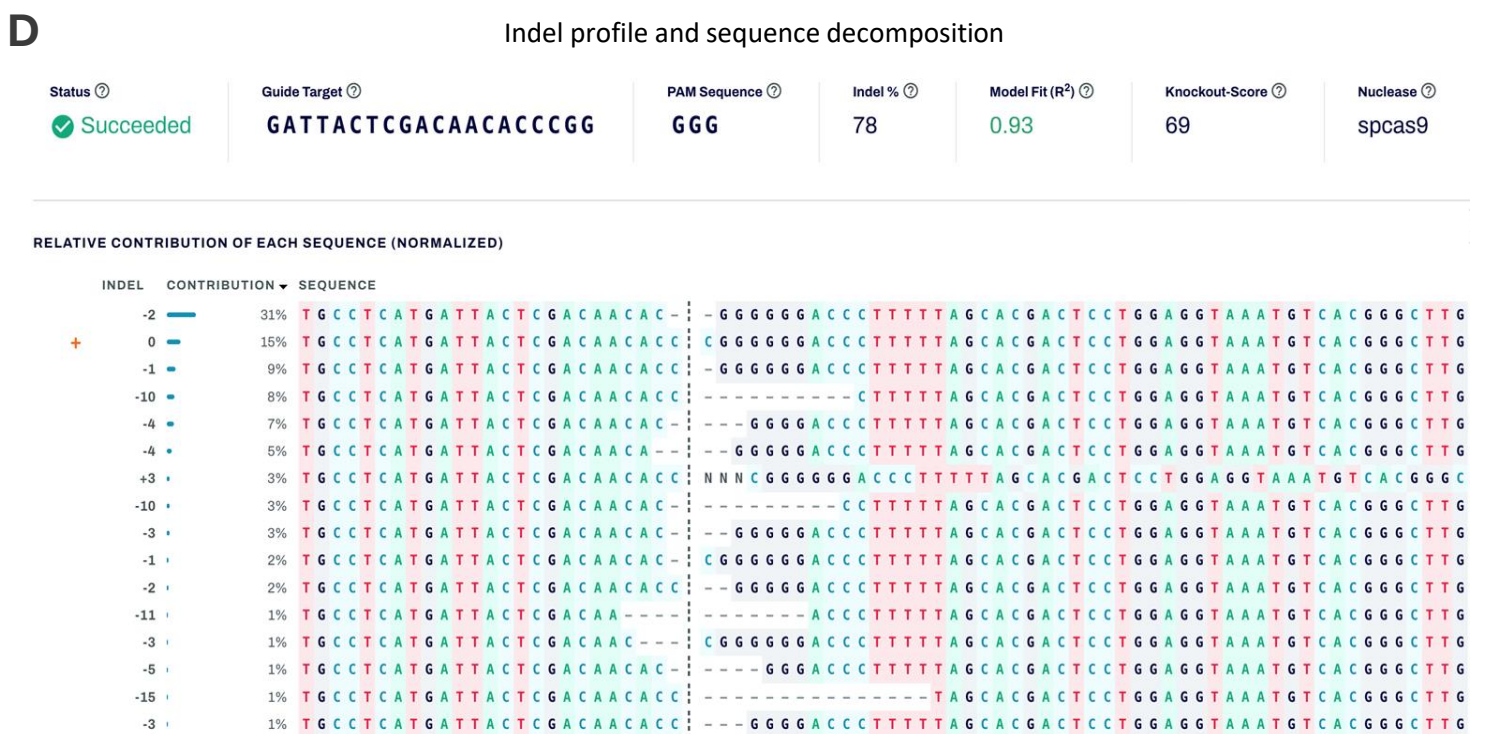
B



C



D



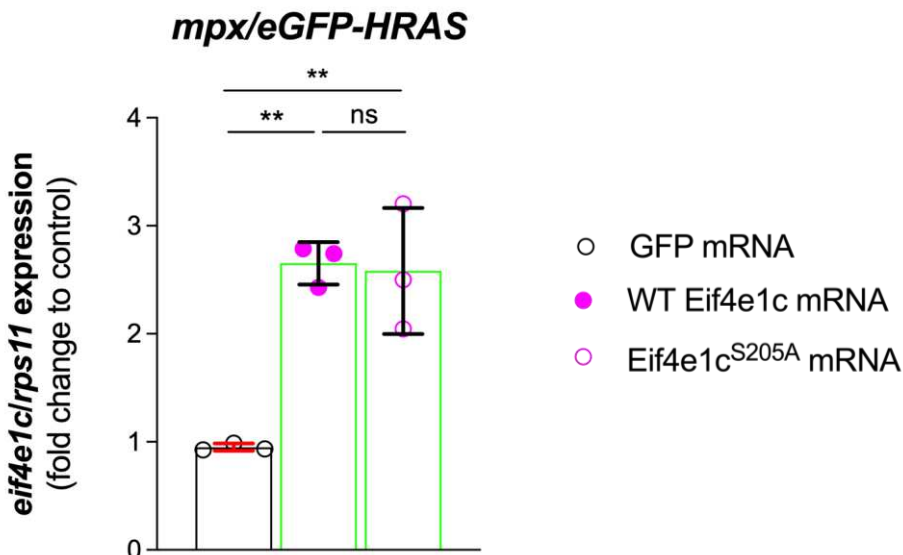
A

ICE analysis

Figure S3

Experiment (crRNA)	Indel %	Model Fit (R ²)	Knockout Score
1 (<i>eif4ebp1</i> crRNA)	56	0.92	51
2 (<i>eif4ebp1</i> crRNA)	87	0.97	80
1 (<i>eif4e1c</i> crRNA 1)	66	0.73	29
2 (<i>eif4e1c</i> crRNA 1)	66	0.75	28
1 (<i>eif4e1c</i> crRNA 2)	66	0.98	23
2 (<i>eif4e1c</i> crRNA 2)	58	0.99	19

B



Supplementary Table S1. Summary of transgenic lines used in this work, genotyping strategies, fluorescent phenotypes, and corresponding fluorescence filters

Transgenic line	Genotyping method	Fluorescent phenotype (responsible transgene)	Fluorescence filter
<i>mpx:eGFP</i>	Fluorescence 	Green neutrophils 	Green (EGFP)
<i>lyz:dsRED</i>	Fluorescence 	Red neutrophils 	Red (dsRED)
<i>mpx:eGFP; lyz:dsRED</i>	Fluorescence 	Green neutrophils (<i>mpx:eGFP</i>) 	Green (EGFP)
		Red neutrophils (<i>lyz:dsRED</i>) 	Red (dsRED)
<i>lyz:mVENUS-NRAS</i>	Fluorescence 	Blue eye 	Blue (ECFP)
<i>lyz:mVENUS-NRAS; mpx:eGFP</i>	Fluorescence 	Blue eye (<i>lyz:mVENUS-NRAS</i>) 	Blue (ECFP)
		Green neutrophils (<i>mpx:eGFP</i>) 	Green (EGFP)
<i>lyz:mVENUS-NRAS; mpx:eGFP; lyz:dsRED</i>	Fluorescence 	Blue eye (<i>lyz:mVENUS-NRAS</i>) 	Blue (ECFP)
		Green neutrophils (<i>mpx:eGFP</i>) 	Green (EGFP)
		Red neutrophils (<i>lyz:dsRED</i>) 	Red (dsRED)
<i>mpx:GAL4</i>	Functional cross	Green neutrophils in progeny (<i>mpx/eGFP-HRAS</i>) 	Green (EGFP)
<i>UAS:eGFP-HRAS</i>	Functional cross	Green neutrophils in progeny (<i>mpx/eGFP-HRAS</i>) 	Green (EGFP)
<i>UAS:eGFP-HRAS; lyz:dsRED</i>	Fluorescence 	Red neutrophils (<i>lyz:dsRED</i>) 	Red (dsRED)
	Functional cross	Green neutrophils in progeny 	Green (EGFP)

		<i>(mpx/eGFP- HRAS)</i>	
<i>mpx/eGFP- HRAS</i>	Fluorescence 	Green neutrophils 	Green (EGFP)
<i>mpx/eGFP- HRAS; lyz:dsRED</i>	Fluorescence 	Green neutrophils <i>(mpx/eGFP- HRAS)</i> 	Green (EGFP)
		Red neutrophils <i>(lyz:dsRED)</i> 	Red (dsRED)

Supplementary Table S2. crRNAs used in this study

IDT name / catalog name	Internal name (study ID)	Target gene	crRNA target site (5'→3')
Negative control crRNA	Control crRNA	non- targeting	IDT #1072544
Dr.Cas9.EIF4EBP1.1.AB	<i>elf4ebp1</i> crRNA	<i>elf4ebp1</i>	GATTACTCGA CAACACCCGG
Dr.Cas9.EIF4E1C.1.AB	<i>elf4e1c</i> crRNA1	<i>elf4e1c</i>	ACGGCTGTTG TGACGACAAG
Dr.Cas9.EIF4E1C.1.AA	<i>elf4e1c</i> crRNA2		ACAACGACCT TGACCGCTAC

Supplementary Table S3. Primer sequences used for PCR and/or sequencing of regions flanking the crRNA target site of each gene

crRNA	Target gene	Primer name	Primer sequence (5'→3')	Primer application	Expected amplicon size (bp)
<i>elf4ebp1</i> crRNA	<i>elf4ebp1</i>	<i>elf4ebp1</i> -crRNA-validation-F	GTCACAAAC GGACAAGGT AAACA	PCR Sequencing	411
		<i>elf4ebp1</i> -crRNA-validation-R	TTTGACCGCG ACATAGCGA	PCR	
<i>elf4elc</i> crRNA1	<i>elf4elc</i>	<i>elf4elc</i> -crRNA1-validation-F	GGTATATATC ACCATTCACC	PCR Sequencing	681
		<i>elf4elc</i> -crRNA1-validation-R	CAGATAGAG GTAAAGTTGG	PCR	
<i>elf4elc</i> crRNA2		<i>elf4elc</i> -crRNA2-validation-F	CTCACCCATT TTGATGGAG CTAAAC	PCR Sequencing	763
		<i>elf4elc</i> -crRNA2-validation-R	GTGGCAGTA TGTAAGTGT GGG	PCR	

Supplementary Table S4. Primer sequences used for RT-qPCR

Gene name	Primer name	Primer sequence (5'→3')	Primer application
<i>rps11</i>	<i>rps11</i> -Fw	ACAGAAATGCCCCTTCACTG	RT-qPCR
	<i>rps11</i> -Rv	GCCTCTTCTCAAAACGGTTG	
<i>EIF4E1C</i>	<i>EIF4E1C</i> -Fw	GAACAGTACATCAAACACCC	
	<i>EIF4E1C</i> -Rv	GGTTTTCTGTCCAACCTTTTG	
<i>mVENUS-NRAS</i>	<i>mVENUS-NRAS</i> -Fw	GCTGTACAAGCTCGAGGGC	
	<i>mVENUS-NRAS</i> -Rv	ATTGTCAGTGCGCTTTTCCC	

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Datasets.zip](#)