Comprehensive studies of mRNA 3'UTR processing during zebrafish early development

（ゼブラフィッシュ初期発生における mRNA の 3'UTR プロセシングの包括的研究）

A DISSERTATION
Submitted to the Graduate School of Life Science,
Hokkaido University
in partial fulfilment of the requirements for the degree
DOCTOR OF LIFE SCIENCE

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2025.9

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ACKNOWLEDGMENTS

First and foremost, I would like to thank Pr Tomoya Kotani for making all of it possible. You gave so much of yourself to help me. I am so very grateful.

Thank you to Pr Atsushi Kimura and Pr Yukako Chiba for accepting to co-direct this thesis and their precious advice.

To past and present lab members Natsumi Takei, Yuki Takada, Keisuke Sato, Wang Jin, Moeko Sakai, Anna Ishii, Ito, Takahiro Sanada, Anyu Suginishi, Liu Wenmo, Dilushi Seralathan, Rinka Ikehara, Shoji Saito and Yuto Kasuya.

Natsumi Takei: showing me around and helping me open a bank account but also simply introducing me to the lab. Yuki Takada: teaching me many experiments with patience and kindness and weird japanese sometimes too. Keisuke Sato: what would Kotani lab be without you? I never knew how to thank you to the extend you have helped all of us, even before we even think of asking you. I am hugging you through words for lack of a better way to do so. Anna Ishii: I am so sorry I talk so much but I am glad that I have found a partner in crime for the US conferences. Madhouse: For laughs and delicious food: Dilushi for comforting me with words and delicious food and Liu for being sassy and entertaining at all times. You guys were my little bubble to be my extra-self.

To my mum and dad, thank you for supporting me even when you don't agree with my choices. I think I have spent my whole life trying to make you proud. I hope it compensates all the times I haven't. Thank you for showing us the world from an early age.

To my big brother, you know that you are the smartest person I have gotten to meet, and

I have always admired how fast your mind works.

To Thomas, for helping me reconnect with myself, teaching me how to laugh again, taking me on that trip, being there through all of it. To Athénais for being one of funniest, warmest person. My not very much alike doppelganger. Our (a)thé times which brought so much laughter. Can't wait to wine and dine in September. (Nihonshu and dine?). To Teva while we never agreed politically after high school where you were my only ally in debate class, we can always share disappointment and despair. Thank you for being my 3 am drunken friend and one of my favourite people (I do mean it). To Loic, somehow whenever I talk with you, I feel like nothing the time has not passed. I know you will succeed whatever you try, be it in Paris or on the other side of the world.

To Marie, for calling me every day when I needed it. You have a crazy life, and I love to hear about it. See you soon in Montpellier for Kaamelott II. To Marion, I can't believe how long it has been. We haven't indeed done our gastronomy tour, but we also haven't participated to Pekin Express. From best friends to best enemies to doing uni together, it's always so natural to be around you. To Nini, med school would have been hell without you, Océane and Louise. For you it always seemed simple, having the best grades of our year while being the sweet you. I knew we would get along well after talking about religion with you.

To all the others in France and everywhere, Sarah, Pauline, Alex, Mikaël, (Chloé for being my alibi to have my last night with Ale thank you thank you), Lola my almost sister-in-law, empathetic and smart.

To **Alle***(xceptional)* for all the ideas, your kindness, and the memories that helped me carry on—*jag älskar dig*. The fact that you chose Toulouse for your PhD after Umeå still baffles me. I owe you for helping me deal with all (and I mean ALL) kinds of frustration,

even from afar, with your ever-calm yet never blasé presence. Also—cheers to real English spelling. Even if the world goes crazy in extremes, it feels reassuring people like you exist.

The people I met in the UK, in particular Gregg and Sophie (congratulations on your wedding) but also all the James, Sophie 2, Susanna…

To Anna K, for being an attentive hear, someone who could understand what I was going through.

To Clara and Phil, the seemingly normal French people of Hokkaido, my home in Sapporo, the people I could be myself and have interesting conversations with.

To Kou, Mitsuha (and husband), Anna, Kiriko, Marie (and Noriko), Takahiro, Max, Miléna, Solène, Sayuri, Clarissa, Lucile, Oriane, Jordan, Koji and your lovely parents, Kuny, Jeanne…

To the gaijin corner (from the original (Biru) to the extended (with Sugo and Fuji) back to the original (with Luisa and Van)). I have to say Van, I am quite impressed to see how strong and independent you are, I do believe you will become someone. Bill (first and last time), even if we disagree on a lot, in the end, you are my only “classmate” and I do like to hear your wisdom, and drink nishonshu together. Luisa, my rock in Sapporo, even when I feel like you would not like to read me describe you like that. You have a beautiful soul, and I love talking with you (sorry). I think I should also apologise for putting you through so much.

Finally, to Najahli, life was unfair to you. I should have been there for you. You were such a talented girl, with strength and empathy. The world needed more of you.

I most likely have forgotten some people, you will be entitled to scream at me when we meet, but only if you can say anticonstitutionnellement backwards.

If I end up in Umeå, I'll call myself Emmanuelle Charpentier, minus the Nobel prize, the talent, the overall aura.

ABBREVIATIONS

APA : alternative polyadenylation

aUTR: alternative UTR

CF: cleavage factor

CPSF: cleavage and polyadenylation specificity factor

CstF: cleavage stimulation factor

cUTR: constitutive UTR

GO: gene ontology

hpf: hour(s) post fertilisation

IPA : intronic polyadenylation

lncRNA: long noncoding RNA

MFE: minimum free energy

miRNA : microRNA

mRNA : messenger RNA

MZT : maternal to zygotic transition

ncRNA: non-coding RNA

NGS: next generation sequencing

PAB: polyadenylate binding protein

PAP: polyadenylate polymerase

PAS : polyadenylation site

PAT assay : poly(A) test assay

Pre-miRNA: precursor miRNA

Puro-PLA: puromycine proximity ligation assay

RPM: reads per million

tRNA: transfer RNA

ULC : UTR length change

UTR : untranslated region

ZGA : zygotic genome activation

GENERAL INTRODUCTION

At very first moments of a zygote's development, maternal mRNAs, inherited from the oocyte, are the only scripts available for protein coding and other biological regulations. Although transcriptional activity has been described as minor at most (Vastenhouw et al, 2019, Chen et al, 2021, Bhat et al, 2023), the egg is not dormant and the growth proceeds in a precisely calibrated manner (Leichsenring et al, 2013, Lee et al, 2013, Kobayashi et al, 2021, Zhou et al, 2023). For each mRNA to be translated when its time has come to serve, multiple cues in the form of motifs lie within its sequence to either recruit necessary actors for its translation initiation, move, or protection (Curtis et al, 1995, Hake et al, 1997). Understanding gene regulation during early development is critical for elucidating the molecular mechanisms that shape embryonic fate. Eggs, being the earliest stage of the developmental cycle, carry a rich diversity of maternal mRNAs that are essential for initiating embryogenesis and guiding subsequent cellular differentiation. As development progresses, the regulation of these mRNAs becomes crucial for ensuring proper spatial and temporal gene expression.

The regulation of mRNA, encompassing processes such as transcription, splicing, degradation, and translation, is not only essential for the maintenance of developmental programs but also for the adaptability of the embryo to changing conditions. Misregulation of mRNA expression can lead to developmental abnormalities, highlighting the importance of understanding these processes in the context of developmental biology (Lopez, 1998, Pikulkaew et al, 2011).

Polyadenylation and mRNA processing are fundamental processes that regulate gene expression at multiple levels, being major actors in cellular development, differentiation, and response to environmental stimuli (Wilkie et al, 2003, Andreassi et al, 2009, Beaudoin et al, 2013, Navarro et al, 2021). One key aspect of this regulation is the influence of the 3' untranslated region (3'UTR), which contains important elements that control mRNA stability, localisation, and translational efficiency. The 3'UTR regulated, particularly, through alternative polyadenylation (APA), which results in the generation of multiple mRNA isoforms with distinct 3'UTR lengths. These isoforms can have completely different properties and expose different sites. For instance, microRNA-binding sites or RNA-binding protein motifs might become accessible, which in turn influence mRNA fate and cellular function. The dynamic regulation of APA, through the selection of different polyadenylation signals (PAS), is pertinent in various biological processes, including cellular differentiation, and disease progression (Tian et al, 2016, Deng et al, 2017, Mitschka et al, 2022, Xin et al, 2023).

microRNAs (miRNAs) are important regulators of mRNA stability and translation. miRNAs bind to the 3'UTR of target mRNAs, often leading to mRNA degradation or translational repression (Valencia-Sanchez et al, 2006, Hu et al, 2012). Studies have also revealed that miRNAs can bind to mRNA without leading to its clearance, resulting in shorter mRNA isoforms that retain translational activity, but exhibit altered stability or localisation (Ito et al, 2011, Iwakawa et al, 2013, Ni et al, 2016). Studies in the early development can be conveniently carried out on zebrafish, with its transparent egg, which allows to easily look at *in vivo* phenotypical changes and fluorescent reporter expression. In zebrafish, the role of miRNAs in regulating APA and mRNA processing

is still underexplored, and the lack of comprehensive miRNA annotations for this model organism presents a significant gap in our understanding.

A number of proteins are involved in the early development. Their expression regulation is coordinated with translation factors such as eIFs, PABCs, CPSFs and the such (Anthony et al, 1991, Mader et al, 1995, Dantonel et al, 1997, Kozlov et al, 2001, Kahvejian et al, 2001, Nag et al, 2007). This regulation could impact major biological events, namely the maternal-to-zygotic transition (MZT), where precise control over gene expression is necessary for its very happenstance. Indeed, Pou5f3, zebrafish orthologue of Oct4, has been extensively studied in the context of the MZT, with evidence pointing at its obligatoriness in the activation of zygotic transcription (Leichsenring et al, 2013, Lee et al, 2013). However, the molecular mechanisms by which Pou5f3 is expressed, its mRNA processing and effects on its own translation, remain only partially understood.

mRNA processing, particularly through 3'UTR length change (ULC), can work as a mechanism to calibrate the cellular response according to the needs of developmental processes. The MZT in zebrafish provides a model system to investigate the regulation of mRNA processing during early development. Furthermore, studies of intronic polyadenylation (IPA), which involves the generation of non-coding isoforms that can regulate gene expression, add another layer of complexity to the regulation of mRNA length and translation (Tian et al, 2017, Singh et al, 2018). The next-generation sequencing technologies, particularly 3'UTR RNA sequencing, have advanced our

ability to study these processes in detail, revealing intricate patterns of shortening and lengthening and their implications for gene expression and cellular function.

This thesis explores the actors guiding upstream and downstream modifications resulting in mRNA activation, focusing on their roles in regulating mRNA processing during early zebrafish development. By examining how CPSF6 behaves in the early development, and how miRNAs potentially interact with 3'UTRs to modulate gene expression, we aim to better understand the mechanisms that control mRNA stability, localisation, and translation during developmental transitions and how in turn, these changes impact protein expression. The findings presented here will shed light on the regulatory networks managing the early development.

Chapter I

pou5f3 mRNA analysis in zebrafish early development.

ABSTRACT

The Maternal-to-Zygotic Transition (MZT) represents a pivotal stage in early zebrafish development, marking the shift from maternal transcript-driven regulation to zygotic genome activation (ZGA). Maternally inherited molecules, including mRNAs orchestrate nearly all biological processes post-fertilization until the ZGA and beyond. Among the key transcription factors implicated in the ZGA, Pou5f3 has been shown to play a critical role. However, the precise mechanisms by which Pou5f3 starts it activity to then initiate the ZGA remain incompletely understood. The 3' Untranslated Region (3'UTR) of mRNAs, though not coding for proteins, is essential for post-transcriptional regulation and influences RNA stability, localisation, and translational efficiency. This chapter challenges the conventional view of mRNA shortening as not a degradation process but a novel regulatory mechanism: mRNA shortening as a driver of translational activation. Using Poly(A) test (PAT) assay, we demonstrated a progressive shortening of the 3'UTR of *pou5f3* and *wnt8a1* from the oocyte stage onward. Analysis of transgenic lines for *pou5f3* 3'UTR hinted towards a higher activity of a shortened *pou5f3*. Furthermore, puro-proximity ligation assays revealed that shorter mRNAs exhibit enhanced translational efficiency. *In situ* hybridisation of *pou5f3* and qPCR of the reporter showed this is not related to mRNA expression number nor their spatial location. These results suggest the existence of a previously unknown mechanism of translational regulation.

INTRODUCTION

Pou5f3 has been extensively studied and is recognised as a crucial factor in the MZT (Leichsenring et al, 2013, Lee et al, 2013) but also in the progression of gastrulation, endoderm differentiation and dorsal-ventral formation (Lunde et al, 2004, Reim et al, 2006, Kotkamp et al, 2014, Nishitani et al, 2015, Perez-Camps, 2016, Inomata et al, 2020, Ducos et al, 2022). Across multiple species, Pou5f3 has been identified as a necessary regulator of early development. In both mice and humans, reduced Pou5f1/Oct4 expression promotes trophoblast and endoderm differentiation (Hay et al., 2004), underscoring its essential role in pluripotency. Furthermore, Pou5f3 is the zebrafish orthologue of Oct4, a transcription factor which has become renowned for its role in cellular reprogramming as part of Yamanaka's factors (Yamanaka, 2008, Zhu et al, 2010, Sterneckert et al, 2012, Takahashi et al, 2016). This association highlights its significance in stem cell biology and potential applications in personalised medicine (Yamanaka, 2008, Takahashi et al, 2016, Bruno et al, 2024).

In early development, the zebrafish embryo is largely transcriptionally silent, with only few transcription factors are being translated before the onset of ZGA. Pou5f3, Nanog, and SoxB1 are among the most abundantly translated factors, and their combined loss results in the failure of approximately 25% of zygotic genes to activate, supporting their central role in MZT (Lee et al., 2013). This study inferred that their high translational activity signifies their critical involvement in initiating the ZGA.

A widely accepted model in developmental biology posits that maternal transcript clearance is a prerequisite for ZGA (Walser et al, 2011, Sha et al, 2019, Sha et al 2020). This theory is supported by the sharp decline in long maternal transcripts from 4 hours post-fertilisation (hpf) onward (Sha et al, 2019, Zhao et al, 2020). The shortening of mRNAs may serve as an additional, or even alternative, regulatory mechanism that facilitates this transition (Kuersten et al, 2003, Mayr et al, 2009, Sonneveld et al, 2020). Furthermore, polyadenylation length is known to influence translational activity; shorter poly(A) tails are often associated with reduced translation but enhanced stability (Bernstein et al, 1989, Subtelny et al., 2014).

The 3'UTR has emerged as a key player in post-transcriptional regulation. This non-coding region at the mRNA's 3' end modulates stability, localisation, and translational efficiency through the presence of regulatory elements such as RNA-binding protein-binding sites and alternative polyadenylation signals (Wilkie et al, 2003, Andreassi et al, 2009, Beaudoin et al, 2013). Structural modifications in the 3'UTR can alter RNA-protein interactions, subsequently influencing translation (Jackson, 1993, Wilkie et al, 2003, Andreassi et al, 2009, Navarro et al, 2021). Our recent findings indicated that *pou5f3* mRNA with a longer 3'UTR formed stable, solid-like granules, which restrict ribosomal access and translation. After fertilisation, the *pou5f3* RNA granules change properties in liquid-like granules, allowing for more efficient translation (Sato et al., 2022). However, molecular mechanisms by which *pou5f3* mRNA is translationally activated remain unclear.

In this chapter, I found that the 3'UTR of *pou5f3* was shaved off of 72 bases from the immature oocyte stage, steadily shortening to reach a point where over half of the transcripts were short at 2 hpf. At the same time, short *pou5f3* 3'UTR showed an effective translation while long *pou5f3* suffered from a delay in translation.

MATERIALS AND METHODS

Danio rerio

The zebrafish (AB strain) were raised in the laboratory, with a light period of 14 hours (from 9 am until 11 pm) and a dark period of 10 hours. The water is maintained at 28°C.

Total RNA extraction

For the embryos, 50 embryos were collected and washed with E3 solution (5 mM NaCl, 0,17 mM KCl. 0,33 mM $CaCl_2$, 0,33 mM $MgSO_4$ and 0,1% Methylene Blue). Oocytes were collected after sacrifice and washed in fish Ringer solution (116 mM NaCl, 1,8 mM $CaCl_2$, 2,9 mM KCl, 5 mM HEPES; pH 7,2). In the case of mature oocytes and immature oocytes, they were treated with 17α,20β-dihydroxy-4-progen-3-one (17α,20β-DP) in PBS (Phosphate-buffered saline) for 3 hours after which only the mature oocytes were selected. After complete removal of the solution, TRIzol (Ambion) was used for RNA extraction.

cDNA synthesis

After extraction of RNAs, 2500 ng of the total RNA sample were used for reverse transcription using Superscript III First-strand synthesis system (Invitrogen) which was used according to the maker's protocol.

PAT assay

Using the extracted total RNA, a P1 anchor primer (5'-P-GGT CAC CTT GAT CTG

AAG C-NH_2-3') was ligated to the polyA tail using T4 RNA Ligase (Takara). Then, using the Superscript II Reverse Transcriptase (Invitrogen), the transcripts were primed using a P1' primer (5'-GCT TCA GAT CAA GGT GAC CTT TTT-3'). This allowed to synthesise the first strand of cDNA. Then, using 2 µL out of the total 10 µL of cDNA as a template and Expand High Fidelity PCR System (Roche), a 1st round of PCR (total volume = 26 µL) was carried out on all extracted RNA samples. From the PCR products, 2 µL were used for a 2nd round of PCR (total volume = 26 µL), The primers for *pou5f3* were as follows:

z-pou5f3-PAT-f1 (5'-TAG ATG TAC TCT TTG TCA GGG TGG-3') and P1' for the 1st PCR

z-pou5f3-PAT-f2 (5'-TGG AGG TCA TGT GCC TTA TCT TTC-3') and P1' for the 2nd PCR.

Regarding *wnt8a1*, the following primer couples were used:

wnt8a-seq-f1 (5'-CGG AAG TTG GAG ATG GAT-3') and P1' for the 1st round of PCR

wnt8a-1208-PAT-f2 (5'-AGA CAC GGA AGC CAT GGT CAT-3') and P1' for the 2nd round PCR.

The products were migrated on agarose gel. The products were furthermore cloned using pGEM-T easy vector (Promega) after which they were sequenced. After reading, shortened reads (shorter than the longest sequence) and full-length reads were classified as "long" and "short" manually.

Whole mount in situ hybridization

Zebrafish embryos were fixed with 4% paraformaldehyde in PBS for 15 hours at 4°C

after which, they were dehydrated with grading concentration of MetOH until 100% for 15 hours at 4°C. GFP RNA probes we synthesised using DIG RNA Labeling kit (Roche) and were used for prehybridization for 15 hours at 65°C. On the 4th day, 2% blocking reagent in PBS was added as well as α-DIG alkaline phosphatase antibody (concentration of 1/2000) (Roche) for 15 hours at 4°C. Coloration was operated at room temperature for 1 to 4 hours depending on the sample batches, until purple was clearly visible, from which stop solution (1M TrisHCL pH 8, 0,5M EDTA, DW) was applied.

GFP protein reporter assay

Using pT2KXIGΔin, the GFP-pou5f3 3'UTR reporter genes were constructed by replacing the Ef1 promoter and SV40 3'UTR with the heat shock promoter and pou5f3 3'UTRs, respectively. Transgenic zebrafish were generated using Tol2 transposon-mediated germline transmission. Three lines carrying the same reporter gene were generated and analysed. All lines carrying the same reporter gene gave equivalent results. Adult female fish were heat-shocked at 38°C for 1 hour for 5 days in a row before mating with non-heat-shocked transgenic males (hsp-GFP-long/short/SV40 3'UTR). Two to three different transgenic lines were used for each type (short 3, long 2, SV40 2). Eggs were collected in the next morning and observed from 0 hpf until 72 hpf under a fluorescence stereo microscope (Leica).

Quantitative RT-PCR

The amounts of mRNA were quantified using a real-time PCR system with SYBR green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. The amounts of the reporter mRNAs and *eIf1α* mRNAs were quantified using primer

sets specific for GFP and eIf1α mRNAs. Primer sets were as follow:

GFP-f 5'-CGACCACTACCAGCAGAACA-3'

and GFP-r 5'-AAATCGCTGCCCTGGTCGTT-3'

zeIf1a-f 5'-GGAGACTGGTGTCCTCAA-3'

and *zeIf1a*-r 5'-GGTGCATCTCAACAGACTT-3'

The level of reporter mRNAs was normalized to that of *eIf1α* mRNA.

Proximity ligation assay

To detect Puro-PLA signals associated with the reporter mRNAs, an anti-GFP rabbit antibody (Sigma-Aldrich, G1544) and an anti-puromycin mouse antibody (Kerafast, 3RH11) were used. The PLA was carried out using the Duolink In Situ Red kit (Sigma) according to the manufacturer's instructions, with some minor adaptations. Briefly, after ribopuromycylation treatment and fixation with 4% PFA in PBS, embryos were incubated in Duolink Blocking Solution for 1 hour at room temperature. They were then incubated for 90 minutes with anti-puromycin (1:300) and affinity-purified anti-GFP (1:50) antibodies, diluted in Duolink Antibody Diluent. Following washes in Buffer A (10 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween-20; pH 6.8), Duolink PLA probes (mouse PLUS and rabbit MINUS; Sigma, DUO092001 and DUO09002) were applied at 1:5 dilution for 1 hour at 37°C. After additional washes, ligation was performed using the supplied Ligase (1:40 dilution in Ligation Buffer) for 30 minutes at 37°C. Signal amplification and labeling with FarRed were carried out for 100 minutes at 37°C. Amplification was stopped by washing with Buffer B (0.2 M Tris-HCl, 0.1 M NaCl; pH 6.8), followed by a final wash in PBS. Embryos were mounted using VECTASHIELD Mounting Medium with DAPI and imaged using a Zeiss LSM 980 confocal microscope.

The number of Puro-GFP PLA sites was quantified using ImageJ.

mRNA reporter assay

SV40 3'UTR and long *pou5f3* 3'UTR sequences were cloned using pGEM-T easy system (SV40 and long *pou5f3* 3'UTR) and inserted into the pCS2-GFP vector using the XhoI and NotI restriction sites. After in vitro transcription with mMESSAGE mMACHINE SP6 kit (Thermo Fisher Scientific), the mRNAs, at a concentration of 300 ng/μL, were injected into 1-cell stage embryos. The eggs were observed for 72 hours after injection.

RESULTS

Shortening of 3' UTRs of mRNAs during Early Development

pou5f3 mRNA has been extensively studied in our laboratory, according to previous studies highlighting the critical role of the poly(A) tail in determining mRNA stability and translation efficiency (Jackson et al, 1990, Passmore et al, 2022). In this context, the PAT assay was employed to investigate the role of the poly(A) tail in the regulation of *pou5f3* mRNA. This method involves the ligation of an anchor to the poly(A) tail, followed by primer extension of the 3' end, which allows amplification of the region, including the poly(A) tail, through a simple PCR-based approach.

Samples were collected at various developmental stages: immature oocytes, mature oocytes, and embryos at 0 hpf, 2 hpf, and 4 hpf, with the MZT expected to begin at around 3 hpf. Although maternal transcripts cannot strictly be discriminated from zygotic ones, zygotic transcription being minimal before 5,5 hpf (Bhat et al, 2023), it is easy to assume most of mRNA to be maternal. PCR amplification products of PAT assay were subsequently cloned into pGEM-T vectors for amplification and sequencing. Two primary transcript isoforms of the *pou5f3* 3'UTR were identified: a long isoform and a shortened isoform (lacking 72 nucleotides compared to the full-length) (Fig 1A, B).

Quantitative analysis revealed that, in the oocyte stage, the long *pou5f3* mRNA accounted for over 70% of the transcripts (Fig 1C). However, its relative abundance decreased steadily over time, reaching its lowest point (10%) at 4 hpf. Conversely, the short isoform increased in prevalence, from representing 30% of transcripts at 0 hpf to

85% at 4 hpf. Despite changes in transcript isoforms, the poly(A) tail length only exhibited lengthening trends across the five time points analysed, when the mRNA was shortened (Fig 1D).

To assess whether this phenomenon was unique to *pou5f3* mRNA, a similar experiment was conducted with *wnt8a1* mRNA, which is thought to play a pivotal role in zebrafish development, particularly in axis formation and dorsal specification (Lu et al, 2011). Results for *wnt8a1* mRNA mirrored those of *pou5f3* mRNA(Fig 1E-G), with full-length transcripts predominating in immature oocytes and short transcripts appearing in nearly all samples by 4 hpf, specifically after 2 hpf. Notably, the poly(A) tail exhibited a shortening at 2 hpf, followed by elongation at 4 hpf, expanding from 12 adenines to over 40.

mRNA Translation Efficiency

To investigate the effect of 3' UTR shortening on translation efficiency, transgenic zebrafish expressing either the long or short *pou5f3* isoforms were analysed (Fig 2A). These models utilised GFP as a reporter protein, with SV40 serving as a positive control. SV40 is a rapidly translated gene, which undergoes no post-transcriptional modifications, providing a useful benchmark for mRNA processing analysis. Transgenic zebrafish were subjected to heat shock, and protein expression was assessed in fertilized embryos (Fig 2B, C ,D and Fig 3A, B).

Fluorescence microscopy revealed that transgenic lines expressing GFP-short *pou5f3* 3'UTR exhibited higher, more intense GFP expression than transgenic lines expressing

GFP-long *pou5f3* 3'UTR. Interestingly, fluorescence was restricted to the yolk, regardless of autofluorescence, in transgenic lines expressing reporter mRNAs with *pou5f3* 3'UTR for reasons that remain unclear, while GFP fluorescence was observed in the animal pole cells of transgenic lines expressing reporter mRNAs with SV40 3'UTR, as expected (Fig 3A, B).

To observe more directly the translational efficiency of reporter mRNAs, a puromycin proximity ligation assay was performed at 0.75 hpf and 2 hpf (Fig 4A, B, C). This technique, which detects translation sites through the incorporation of puromycin into newly synthesized proteins, showed that the reporter mRNA carrying short *pou5f3* 3'UTR was more efficiently translated than the reporter mRNA carrying long *pou5f3* 3'UTR at both time points. No significant difference was observed between the transgenic lines with short *pou5f3* 3'UTR and the ones with SV40 3'UTR at 2 hpf (Fig 4B).

mRNA Expression in Relation to Transcript Length

Whole mount in situ hybridization was carried out using a *GFP* antisense RNA probe, a *pou5f3* RNA probe as a positive control, and a *GFP* sense RNA probe as a negative control. The experiment was repeated with varying parameters, including combinations of wild-type and transgenic males and females, as well as different fixation times ranging from 2 hpf to 27 hpf (Fig 5A and C). At 2 hpf, GFP mRNA expression levels were comparable across all transgenic lines (Fig 5A). Staining was uniform across the cytoplasm, with minimal labelling of the yolk. The expression levels of reporter mRNAs were further examined by quantitative PCR at 2 hpf which confirmed that GFP

mRNA levels were similar in both transgenic lines expression GFP with short and long *pou5f3* 3'UTR (Fig 5B). At 27 hpf, GFP mRNA had a stronger expression in lines with a transgenic mother compared to the ones with wild-type mother mated with a transgenic father showing the maternal effect to be more lasting than it is widely known to be (Fig 5C).

GFP mRNA Reporter Assay

In order to see whether the extra 72 nucleotides generally hinder the mRNA's translation, a GFP mRNA reporter assay was conducted. In vitro-synthesized GFP-*pou5f3* long 3' UTR and GFP-SV40 mRNAs (Fig 6A) were injected into 0 hpf embryos to assess translation efficiency at the earliest stages of development. Embryos injected with the GFP-*pou5f3* long 3'UTR mRNA consistently exhibited weaker GFP fluorescence compared to those injected with GFP-SV40 3'UTR (Fig 6B). This result suggests that the translation of GFP-*pou5f3* long 3'UTR mRNA is at least partially repressed in embryos.

miRNA Binding Sites

The potential impact of the deleted sequence in the shorter *pou5f3* isoform was explored by searching for destabilising elements, such as UA-rich sequences, AUUUUA motifs, regulatory protein binding sites, and miRNA binding sites. Previous research has highlighted the importance of these sequences in the regulation of mRNAs during the MZT (Shyu et al, 1991, Wellington et al, 1993, Chen et al, 1995, Ruiz-Echevarria 2001, Vejnar et al., 2019). Predictive tools revealed a potential miRNA (expressed in the oocyte, cf chapter 3) binding site in the long 3' UTR of *pou5f3* mRNA, which is absent

in the shorter isoform, suggesting a possible mechanism through which miRNA-mediated regulation contributes to the observed translational control (Fig 6C).

DISCUSSION

Pou5f3 is known to be an essential transcription factor during the development, including the MZT, gastrulation, cell differentiation and body patterning (Lunde et al, 2004, Nishitani et al, 2015, Inomata et al, 2020, Sukparangsi et al, 2022, Maekawa et al, 2024). Previous studies have shown that Pou5f3, along with SoxB1 and Nanog, are the most translated transcription factors prior to the MZT. Knocking out these factors results in a failure of zygotic activation in 75% of genes, which leads to developmental arrest (Lee et al., 2013). The ZGA is a crucial step for the progression of development for many animals (Svoboda, 2018, Chen et al, 2019).

The prevailing hypothesis surrounding maternal mRNAs during the MZT is that the activation of zygotic transcription is tightly linked with the clearance of maternal mRNAs (Giraldez et al., 2006). This suggests that the presence of zygotic genes is necessary for the degradation of maternal transcripts. My results from the PAT assay showed that the mRNAs encoding Pou5f3 and Wnt8a1 underwent 3'UTR shortening before the MZT, with various intermediate forms of mRNAs, ranging from a shorter 3'UTR (lacking 72 nucleotides) to full-length 3'UTRs. This observation suggests the existence of a dynamic process of mRNA shortening rather than a simple metabolic degradation.

Initially, sequencing of *pou5f3* and *wnt8a1* PAT products revealed that the shortening occurred in the 3'UTRs of these mRNAs, which followed polyadenylation. Previous studies have shown that cytoplasmic polyA polymerases can re-extend the polyA tail

(Subtenlny et al., 2014), suggesting that exonucleases might be involved in the degradation of transcripts, with a potential re-extension occurring afterward. *pou5f3* exhibited polyA tail elongation in the short mRNA and *wnt8a1* showed a clear shortening of the polyA tail at 2 hpf, followed by elongation at 4 hpf.

The significance of this shortening was further explored by analysing the relative abundance of short and long forms of *pou5f3* mRNA. Around 2 hpf, the abundance of short and long forms of *pou5f3* was roughly equal. At 4 hpf, the abundance of short forms of *pou5f3* drastically increased. This is consistent with the decrease in longer forms of *pou5f3* post-0 hpf, as they are eventually processed into shorter transcripts.

Using GFP fluorescence as a marker of translation, I observed that short *pou5f3* transcripts led to more GFP expression in transgenic zebrafish embryos, especially after 3.5 hpf. The GFP was primarily localized in the yolk, possibly indicating altered protein tagging, although the mechanism remains unknown. These findings suggest that short *pou5f3* mRNAs are translated more efficiently.

I also employed a puromycine-proximity ligation assay (PLA) to track the translation of short and long *pou5f3* mRNAs. The results revealed no significant difference in translation between reporter mRNAs carrying short *pou5f3* 3'UTR and SV40 3'UTR whereas the translation of reporter mRNAs carrying long *pou5f3* 3'UTR was significantly repressed. This might be explained by physical restrictions on the translation of reporter mRNAs carrying long *pou5f3* 3'UTR mRNAs, as these sequences may form aggregates that hinder translation (Sato et al. 2022). Studies have shown the

presence of stress-like granules in zebrafish embryos, which affect translational activity depending on their physical state (Sato et al., 2022). These granules may restrict translation of long 3'UTR mRNAs like endogenous *pou5f3* mRNA, while short mRNAs are free to form liquid-like granules that allow translation.

The findings suggest that shorter *pou5f3* mRNAs are more translationally active. Although the mechanism of translation repression for long *pou5f3* remains unclear, potential explanations include the presence of destabilizing sequences, protein binding sites, or miRNA binding sites. To explore the potential involvement of miRNAs, I predicted conserved miRNA seed sequences in the 3'UTR of *pou5f3*. While the database was outdated, it is possible that other binding sites are not yet registered.

Finally, it is possible to hypothesize that the shortening of the 3'UTR in mRNAs, like *pou5f3* but also *wnt8a1*, have a major role in the storage or translation regulation of mRNAs. However, at this point, it was still unclear whether this was an isolated event or a more widespread phenomenon, that would represent a novel mechanism of translational control during early zebrafish development.

Chapter II

mRNA processing throughout zebrafish transcriptome

ABSTRACT

During the first hours of zebrafish development, transcripts and proteins are maternally inherited, stabilised, and primed for use when required. The untranslated regions (UTRs) of mRNAs, particularly the 3'UTR, play a crucial role in post-transcriptional regulation by modulating localisation, translational efficiency, and degradation of mRNAs. Changes in 3'UTR length, for example those driven by alternative polyadenylation (APA) or polyadenylation site (PAS) selection, can alter binding sites for proteins and non-coding RNAs (ncRNAs), including miRNAs. Previously, we demonstrated that Pou5f3, a key transcription factor for the maternal-to-zygotic transition (MZT) and later development, undergoes a 72-nucleotide shortening before zygotic genome activation (ZGA), leading to its translational activation. Expanding on this, I performed 3' end RNA sequencing and computational analysis of over 9,000 expressed genes, revealing both lengthening and shortening of thousands of transcripts. I classified the processing into 3 categories and analysed the differences between class I and class II mRNA using gene onthology (GO). Notably, the shortening and lengthening started actively before ZGA, and a broader diversity of these changes emerged between 2- and 4-hours post-fertilization (hpf). I also found the use of PAS within upstream introns. Additionally, I identified hundreds of previously unannotated 3'UTRs, underscoring the extensive and dynamic nature of transcript processing. These results reveal novel mechanisms of mRNA regulation, highlighting their consequential impact on early developmental processes in zebrafish.

INTRODUCTION

Early development is a complex and highly regulated process through which fertilized eggs develop into organized embryos. Transcription being silent shortly before fertilization until the ZGA, early development is directed by mRNAs and proteins stored in eggs. To understand the molecular mechanisms underlying the progression of development, many transcriptome analyses have been performed, particularly in zebrafish (Aanes et al, 2011, Aanes et al, 2013, Harvey et al, 2013, White et al, 2017, Farrel et al, 2018, Wagner et al, 2018, Lawson et al, 2020, Bhat et al, 2023, Fishman et al, 2024). Those studies mainly focused on steady-state mRNA levels in a long time frame of development. On the other hand, translational states of mRNAs were analyzed by isolation of ribosome fractions in the early development of zebrafish and mouse (Winata et al, 2018, Zhang et al, 2022, Xiong et al, 2022). Those studies showed that two to three thousand of the more than ten thousand mRNAs that have accumulated in vertebrate eggs are translationally repressed and are translated at appropriate times and places after fertilization. In addition, although ZGA has been shown to be initiated at around the 1000-cell stage in zebrafish (Kane et al, 1993, Harvey et al, 2013), recent studies have shown that maternal mRNAs are dominant for a long period beyond ZGA even at the gastrula stage (Bhat et al, 2023, Fishman et al, 2024). Therefore, post-transcriptional regulations of maternal mRNAs are fundamentally important not only for the progression of processes before ZGA but also for the progression of all of the early developmental processes.

mRNAs contain in both ends untranslated regions which, although not part of the script for protein translation per se, is very much a central element for the mRNA's spatial localisation, degradation and translation activation or repression. Here, we mostly focus on the 3'UTR. Indeed, the 3'UTR contains binding sites for proteins and non-coding RNAs to act as an activator or hinder translation whether directly or through the recruitment of another actor (Wilkie et al, 2003, Andreassi et al, 2009, Tian et al, 2017).

Each level of structure of mRNAs is carefully regulating its expression. The primary structure creates simple binding sites that are already influenced by their electrical charges and interactions giving secondary and tertiary structures (Conn et al, 1998, Holbrook et al, 2005). Proteins binding to the mRNA would bind to other proteins themselves creating a bundle of multiple components hindering the access of ribosomes, tRNA, translation elements to their sites and therefore, making translation challenging (Draper et al, 1999). By deleting a few bases or adding some others to the UTRs through a change of polyadenylation signal (PAS) for instance, the structure of the mRNA can change completely, making it either translationally active, unstable or unaccessible (Kozak, 1988). This phenomenon is commonly studied in the shape of what is referred to as "granules" (Anderson et al, 2009, Lavut et al, 2012). What is more interesting, this length alteration can lead to a butterfly effect with cascades being starved or fuelled (Lewandowski et al, 2002, Hirata et al, 2003, Miura et al, 2013, Bentley, 2014).

One motif that can define mRNA's size is PAS (Colgan et al, 1997, Beaudoing et al, 2000, Zarudnaya et al, 2003, Tian et al, 2012). They are varied and numerously located in the 3'UTR with more or less strength. A strong PAS is a PAS that tends to be selected even while another is located around, which is helped by surrounding sequences (Chao et al, 1999, Legendre et al, 2003, Stroup et al, 2023). When the strongest proximal PAS is selected, it creates an isoform with what is referred as "constitutive UTR"(cUTR), which is the shortest possible UTR known. On the other hand, other more distal PASs generate "alternative UTRs" (aUTR). These 3'UTR are called alternative polyadenylation (APA). Since the selection of a PAS defines mRNAs' resulting in aforementioned regulation types (three-dimensional structure, hindrance, charges and other physical congruences), different RNA- and polyadenylation binding proteins bind to different motives. (This will be discussed in the next chapter.)

Intronic polyadenylation (IPA) is a murky area when it comes to APA studies. The phenomenon is known to exist and be of relative importance (Thomas et al, 2007, Singh et al, 2018, Lee et al, 2018) yet has been out of focus for long enough that it can be disregarded. IPA is a regulation mechanism of genes in the development which generates either no protein or possibly a "negative effect" peptide (Lou et al, 1998, Hoque et al, 2013, Sun et al, 2024). The use of which APA [intronic or exonic (possibly unreferenced 3'UTR)] impacts the translation of said mRNA to give a functional protein, with a role outside of gene regulation or barely a feedback peptide (Hoque et al, 2013).

Results of previous studies have shown shortening and lengthening to not be necessarily linked to a change of APA (Wilusz et al, 2010, Malka et al, 2017). Studying isoform changes with and without the PAS factor can be interesting for understanding the underlying factors for this happenstance (different types of processing according to what can bind to what sequence) and the types of RNA targeted (size-wise for instance).

RNA sequencing has evolved over the years to reach peak efficiency to this day in what has been dubbed “next generation sequencing” (NGS). These technologies encompass Illumina, Oxford nanopore and Pacbio (Weirather et al, 2017). As opposed to Sanger sequencing, large amounts of data can be gathered from a small collection of pooled and tagged RNAs. Illumina tends to be used for shorter reads and is more precise. For a better collection, when focusing on 3'UTR sequencing, 3'UTR RNA sequencing is the most adequate way to get plethora of accurate reads of this specific region. However, a drawback of the method is related to how most viewers (including IGV) are built and coded for full-length RNA sequencing which, when performed using an Illumina kit, works like a puzzle by assembling the pieces to create one long transcript to analyse. Since in our case one read is equivalent to one transcript, this was a challenge that had to be faced. At the beginning of our research, we were counting the reads manually. After carrying out 3'end-sequencing, to overcome these hurdles, I used computational methods, which allowed me to confirm our findings at a more significant scale. Similarly, although mRNAs tend to be well annotated in species like human or mice, zebrafish still has uncharted territories in the transcriptome to be discovered. When it comes to the first hours of its development, this truth is even more striking with

research usually focusing on the after part of the ZGA (Lawson et al, 2020). There was a need to address this issue as well.

In this chapter, I described new types of mRNA 3'UTR shortening or lengthening, including up to the intron in some cases. I also annotated over one thousand of new 3'UTR. I characterise 3'UTRs in the early development. I finally described each class of shortening's difference and their potential fate.

MATERIALS AND METHODS

RNA Extraction

Total RNA was isolated from zebrafish embryos obtained from distinct mating pairs, with 50 eggs collected per pair at three developmental stages: 0 hours post-fertilisation (hpf), 2 hpf, and 4 hpf using the QIAGEN RNeasy Mini Kit (QIAGEN, Cat. No. 74106), according to the manufacturer's instructions. To ensure experimental reproducibility, three independent biological replicates were processed for each time point. Embryos were homogenized in RLT buffer (in kit) with β mercaptoethanol, and RNA was subsequently purified. The quality and integrity of the extracted RNA were assessed using a Nanodrop spectrophotometer (Thermo Scientific), with samples retained for further analysis only if their A260/A280 ratio exceeded 2.0 and their A260/A230 ratio was above 2.0.

Illumina library preparation

For 3'end-RNA sequencing, libraries were generated using the Lexogen QuantSeq 3' mRNA-seq Library Prep Kit (Lexogen, Cat. No. 015.96) for Illumina sequencing, adhering to the standard protocol provided by the manufacturer. The workflow involved oligo(dT)-primed reverse transcription of polyadenylated RNA, followed by second-strand synthesis and adapter ligation. To optimize PCR amplification, an SYBR Green-based qPCR assay was performed to determine the ideal cycle number for endpoint PCR and prevent over-amplification. When the endpoint PCR was performed, a 10-second elongation time was chosen for short and therefore accurately readable read sets. The quality and size distribution of the constructed libraries were evaluated using the

TapeStation High D1000 system (Agilent Technologies), ensuring fragment lengths met the expected range before proceeding with sequencing.

Illumina library sequencing

Library sequencing was outsourced to Macrogen Inc. and executed on an Illumina HiSeqX Ten platform using a 150-base pair (bp) paired-end sequencing strategy. A target sequencing depth of 30 million reads per sample was established to provide adequate coverage for subsequent analyses. Quality control of raw reads was conducted using fastp (version 0.23.4) to remove adapter sequences, low-quality bases, and short reads. The processed reads underwent further quality assessment using FASTQC (version 0.11.8), with problematic reads either trimmed or excluded. Reads were aligned to the zebrafish genome (GRCz11) using STAR (version 2.7.9a) with default alignment parameters. Mapping efficiency was evaluated from STAR output logs, pairing each sample to its most resembling counterpart.

APA Analysis

APA site identification and quantification were conducted using APAlyzer (version 1.0). The analysis was performed on two independently prepared sets of high-quality sequencing libraries, assessed based on read depth, Q30 scores, and mapping efficiency. Comparative analyses of 3' UTRs were conducted across the different developmental stages to examine differential APA site usage. The ThreeMostPair function was utilized to generate 3' end read files, while reference files were prepared using the Ensembl annotation dataset (GRCz11.110) and PAS2GEF. Reproducibility checks were carried out on comparable sets from each time point before compiling data into one

comprehensive dataset.

3' UTR annotation

Novel 3' UTR regions were identified using the peaks2utr pipeline, which mapped read clusters to the zebrafish genome (GRCz11) to detect previously unannotated regions. The Ensembl zebrafish 3' UTR annotation file (GRCz11.110) served as the reference for comparison. Each sample from each time point was ran using the same options namely: override (overrides annotated UTR when new UTR is detected) and 3000 nucleotides being set as the maximum distance from the already annotated UTR. This value was chosen based on previous studies that relied on the length of zebrafish mRNAs being on average over that of other species. Additionally, previously published 3' UTR datasets, such as those from Lawson et al. in 2020, were incorporated into the analysis to facilitate comparative annotation and validation of newly identified 3' UTRs.

Shortening analysis

To identify 3'UTR shortening events, gene expression data were analysed using the Change Point algorithm, which detects significant shifts in transcript length based on read distribution across time points. This analysis was conducted both on individual samples and combined biological replicates to ensure reliability. The minimum and maximum heap size were changed to maximise data analysed to 24go and 32 respectively. The Ensembl zebrafish 3' UTR annotation file (GRCz11.110) served as the reference for comparison. The minimum number of reads to support each region was set to 20. The earliest stage was set as control and the mode selected was “shortening”. The FDR level was set to 0,05, and the hypothesised odds ratio to 2.

GO analysis

Gene ontology was carried out using ShinyGO version 0.82 from South Dakota state university and with a false discovery rate cutoff of 0,05 and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) version 12.

RESULTS

A ubiquitous shortening of mRNAs' 3'UTR before the ZGA

The 3'UTR is highly dynamic, exhibiting significant variability across physiological and pathological contexts, cell types, and, as my study focuses on, developmental stages. I performed 3'end-RNA sequencing using RNAs from embryos at 0 and 4 hpf (Fig 7A and B). RNA sequencing data revealed that the shortening previously characterised extends beyond *pou5f3*, with one third of analysed genes displaying similar patterns of 3'UTR shortening during the critical window of 0 to 4 hpf. In our previous study, we demonstrated that over 40% of transcripts exhibited shortening based on manual read counting of fewer than 600 transcripts (Takada et al, 2023)

While analysing the 3'ends, we noticed that various types of shortening could be found. We classified the types of shortening into three categories (Fig 7C). The first one is a simple loss of several to a hundred nucleotides (Class I; Fig 7C, left), similar to the shortening of the *pou5f3* 3'end (chapter I). The second is a shortening of a long region from hundreds to thousands of nucleotides (Class II; Fig 7C, middle). The third is the use of an intron or a more proximal exon for the shortened 3'end with a polyadenylated tail (Class III, Fig 7C, right). In all classes with varying instances, there are noticeable differences between genes, for example, those with partial reduction of long mRNAs and increase in shorter mRNAs and those showing an absolute disappearance of long mRNAs. The increase in intronic 3'UTRs in Class III may be due to degradation of long variants and/or increase in polyadenylation of the short variant, because the short variant could be detected at the time point of 0 hpf in some cases. This is consistent with

the results of a previous study showing preferential use of intronic 3'UTRs in maternal mRNAs (Ulitsky et al, 2012).

3'end-RNA sequencing generates cDNAs consisting basically of 100-150 nucleotides (Fig. 7A). Almost all reads indicate the 3'ends of individual mRNAs in this analysis. Traditional genome viewers, such as IGV, are not optimized for 3' end sequencing, often generating artificial long reads that obscure the detection of short 3'UTRs (Fig 7B and 8A). This makes manual counting of the reads difficult. In addition, more systematic analyses were required for understanding all of the 3'UTR changes. To achieve comprehensive analysis with an applicable and systematic approach, we attempted some computational analyses.

In this study, we generated two additional samples from embryos at 0 hpf and a 4 hpf as well a set of three samples at 2 hpf (Fig 8B, C,D). Samples with high Q30 values and a sufficient yet balanced read count were selected to minimize experimental and transcript biases (Fig 8C, D). After confirming reproducibility (Fig 8B), we then merged-mapped trimmed samples to get more comprehensive data.

To systematically assess 3'UTR length dynamics, we employed what will be referred herein as “Change Point” (Wang et al, 2014), a computational tool designed to identify shifts in transcript length between conditions. Until now, our approach relied on manual read counting and statistical tests, which, despite their utility, were labour-intensive and prone to human error, particularly when dealing with thousands of overlapping reads. Change Point identified shortening in approximately 36% of analysed transcripts

(making it 3231 out of 8907 genes analysed between 0 and 4 hpf) (Fig 9A). This automated approach provides a more robust and scalable method for investigating 3'UTR dynamics during early embryogenesis.

When getting into the detail of 0 hpf to 4 hpf shortening, it was interesting to note that while many events happened from 2 hpf to 4 hpf (3397 transcripts out of 9486) in a similar fashion to 0 hpf vs 4 hpf, the number of mRNA showing a change of isoform was more significant from 0 hpf to 2 hpf with 5265 out of 11507 (Fig 9A). While useful, this tool is only capable of analysing annotated 3'UTRs and, therefore, only class I or II shortenings happening within the 3'UTR (Fig 9B). It is noteworthy that only 1269 genes are both shortened from 0 hpf to 2 hpf and further processed from 2 hpf to 4 hpf (Fig 9C) hinting towards different mechanisms and/or groups of RNA processing but highlighting the temporal nature of mRNA regulation.

Computational Analysis of APA Dynamics

To achieve a comprehensive and systematic analysis of 3'UTR changes, I employed APAlyzer, a computational tool designed for APA analysis using RNA sequencing data. APAlyzer allows for the identification of both 3'UTR and IPA events across species (Wang et al, 2020).

Alternative 3'UTRs are typically classified as either “constitutive” or “alternative” based on length. My data indicated that 3'UTR utilization varies significantly between early developmental stages. Additionally, I observed the use of intronic alternative polyadenylation sites, independent of developmental stage. Some putative 3'UTRs, not

previously annotated in databases, appeared as extensions of known UTRs, intronic sequences, or novel exonic regions, necessitating further analysis.

I analysed three biological replicates per time point at 0, 2, and 4 hpf, revealing dynamic changes in 3'UTR length throughout early development (an example is shown in Fig 9A). Then, samples were merged and analysed to get the most data possible. At 0, 2, and 4 hpf, 9,356, 11,993, and 7,613 transcripts were detected, respectively. APAlyzer identified significant APA events in 738 mRNAs between 0 and 2 hpf. Among these, 198 exhibited 3'UTR shortening, while 215 showed 3'UTR lengthening (adjusted p-values < 0.05) (Fig 10A, C). Notably, *hmga1a* mRNA displayed an increased proportion of short 3'UTR isoforms, whereas *jupa* mRNA exhibited an increase in long 3'UTR isoforms (Fig 10G). The remaining 325 mRNAs maintained stable APA patterns. IPA changes were similarly prevalent, with 71 mRNAs exhibiting 3'UTR shortening and 70 displaying lengthening (Fig 10B,D).

Between 2 and 4 hpf, APAlyzer detected a similar trend, albeit with greater diversity in 3'UTR changes (Fig 11A,B,C,D,E,F). For example, *ell2* mRNA showed an increase in short 3'UTR isoforms at 4 hpf, whereas *cdc20* mRNA exhibited an increase in long 3'UTR isoforms (Fig 11G).

Functional Implications of APA Dynamics

APA is a key mechanism regulating mRNA stability, processing, localization, and translation. Shortened 3'UTRs often correlate with translational activation, while longer 3'UTRs confer mRNA stability and translational repression (Tanguay et al, 1996).

My analysis using APAlyzer revealed a previously unknown trend of decreased distal PAS (dPAS) usage from 0 to 4 hpf, favoring more proximal PAS (pPAS). Genes exhibiting this shift predominantly encoded proteins involved in RNA splicing, mRNA processing, translation activation, and intracellular localization (Fig 12). On the other hand, genes increasing dPAS usage tend to encode proteins involved in translation repression, mRNA degradation, and regulatory processes, including RNA splicing. This suggests a coordinated transition in mRNA processing machinery during early development.

Notably, between 2 and 4 hpf, a clearer pattern emerged, with strong differences of isoform choice, reinforcing that most 3'UTR remodelling occurs later in early embryogenesis (Fig 12B). In contrast, genes exhibiting 3'UTR shortening or lengthening from 0 to 2 hpf are primarily involved in mRNA metabolism and expression regulation, indicating a fine-tuning of gene expression before ZGA (Fig 12A).

IPA also appears highly active during early development. Genes undergoing IPA predominantly encode regulators of transcription factor activity, RNA binding proteins, and factors involved in domain binding activation. These findings highlight the intricate regulatory landscape governing early zebrafish development.

A vastly unexplored landscape

When analysing my data sets, a striking number of genes displayed visibly extended 3'UTRs or the use of an alternative-yet-not-registered 3'UTR, either intronic or exonic. Alternative 3'UTRs, are common in genes undergoing physiological changes, such as during development, or pathological conditions like cancer, highlighting their role in regulatory plasticity (Tian et al, 2017, Masamha et al., 2014). In many model species outside of the commonly studied mouse and human, incomplete gene annotation is not uncommon. This is particularly true for 3'UTRs, where a lack of annotations can make it difficult to understand gene regulatory processes and expression patterns. peaks2utr addresses this issue by accurately annotating transcriptomes (Haese-Hill et al., 2023).

Notably, *Danio rerio* typically expresses longer transcripts, and it is not uncommon for 3'UTRs to span thousands of bases. For this reason, the gap between a readily annotated UTR and a possibly novel UTR was set at 3000 bases in the peaks2utr analysis, extending already annotated UTRs when possible. At 0 hpf, peaks2utr annotated 1026 new 3'UTRs (Fig 13B). This number decreased to 633 at 4 hpf, which aligns with the fact that the transcriptome of early-stage embryos has been less studied.

A similar study by Lawson et al. (2020) used different pipelines to generate a more accurately annotated zebrafish reference file. By incorporating their work, we reduced the number of newly discovered 3'UTRs to 467 at 0 hpf (Fig 13B). However, a significant number of newly annotated UTRs remained, likely because Lawson et al. focused on embryos well beyond 4,5 hpf (hence why the number decreases to 194 and 121 at respectively 2 and 4 hpf), whereas our study targeted earlier developmental

stages. This discrepancy highlights the gaps that still exist in zebrafish transcriptome annotation.

In addition to identifying new 3'UTRs, I also analysed the total number of 3'end peaks detected by peaks2utr. Across all time points (0, 2, and 4 hpf), more than 35000 peaks were consistently detected, with no significant fluctuations (Fig 13A). Given that the total number of mRNAs detected in our RNA sequencing was approximately 9400, 12000, and 7600 at 0, 2, and 4 hpf, respectively, it is possible to infer an average of around 3 distinct 3'UTRs per gene. A more detailed count of individual mRNAs confirmed that maternal transcripts possess an average of 2,8 distinct 3'ends (Fig 13C). Peaks2utr allowed for detecting not only new 3'UTR withing the CDS, but also extend existing 3'UTR which can be seen in Fig 13D. These findings suggest that most maternal mRNAs harbour multiple 3'UTR variants, contributing to transcriptome complexity and post-transcriptional regulatory diversity.

Types of genes shortened per class

Class I and II mRNAs were further analysed and a GO analysis revealed Class I mRNA carried a longer 3'UTR, on average, than their class II counterparts and in general, of zebrafish transcripts (Fig 14A, B). Class I mRNAs were also involved in transcriptional pathway (Fig 14C, D) while class II, on top of positive feedback for gene expression (Fig 14E), were also involved oocyte maturation or wnt pathways. It is noteworthy that class II UTRs also had, on average, a bigger number of isoforms compared to the rest of the transcriptome (Fig 14F).

DISCUSSION

In chapter I, I discussed the shortening of a specific mRNA which, when translated, works as a key maternal factor for the activation of the zygotic genome and the progression of development. After a transcriptome-wide analysis, my study provided new insights into the intricacies of post-transcriptional regulation during early zebrafish development, particularly through the dynamic changes in 3'UTR lengths. The regulation of maternal mRNAs is fundamental for orchestrating developmental processes after fertilisation, yet the molecular mechanisms promoting these processes remain only partially understood. By comprehensively analysing 3'UTR length changes in early-stage embryos, I demonstrate three phenomena. First, thousands of mRNAs underwent alterations in their 3'UTR lengths during early development. Second, these changes encompassed both shortening and lengthening including intronic polyadenylation. Thirdly, many mRNAs exhibited dynamic shifts in 3'UTR length between 2 and 4 hpf and they were more common before 2 hpf.

Although changes in mRNA levels have been widely studied, our findings highlight an additional layer of regulation wherein variations in 3'UTR length influence translational control. This suggests that early embryonic development is not solely dictated by transcript abundance but also by the fine-tuning of mRNA stability and translational efficiency through 3'UTR modifications.

The length and sequence composition of mRNAs are determined by nuclear processes such as splicing and 3' cleavage and polyadenylation. Extensive studies on

other species have shown that translationally repressed mRNAs initially possess short poly(A) tails, which are elongated prior to their activation through cytoplasmic polyadenylation (Simon et al, 1996, Radford et al, 2008, Lin et al, 2009, Cui et al, 2013). Similar regulatory mechanisms have been observed in zebrafish early development (Winata et al, 2018, Vastenhouw et al, 2019). On the other hand, poly(A) tail shortening is often associated with mRNA degradation. In this chapter, I found that more than three thousand mRNAs shortened their 3'end sequences in early developmental stages (Figs. 2 and 5). These partial shortenings of 3'ends were also found in human embryos in mitotic cleavage stages (Liu et al, 2023), although their biological significance remains unclear. We previously reported that shortening of approximately 70 nucleotides of the *pou5f3* 3'end directed translational activation of mRNA, promoting early developmental processes (as per chapter I). In this process, proteins binding to *pou5f3* mRNA were dynamically changed from those repressing translation to those activating translation (Takada et al, 2023). Although the relationship between the 3'end shortening and translational control in individual mRNAs should be addressed in future studies, it most likely directs translational activation of mRNAs stored in eggs as dormant forms.

The molecular processes driving 3' end shortening remain unclear. One plausible mechanism is exonucleolytic degradation, as opposed to endonuclease mediated cleavage. Additionally, different mechanisms may be responsible for the shortening observed in distinct mRNA classes. These could include exonucleolytic trimming, targeted endonuclease cleavage, or selective degradation of transcripts with longer

3'UTRs. Given the scale of these changes, it is likely that multiple overlapping pathways contribute to 3'UTR regulation during early development.

Interestingly, I also identified hundreds of mRNAs that underwent 3'UTR lengthening during early embryonic stages. While the onset of ZGA occurs shortly before 4 hpf, most of these lengthening events are unlikely to be driven by newly transcribed zygotic mRNAs, given the low transcriptional activity during this period (Bhat et al, 2022). Indeed, the zygotic transcription activity stays under 10% before 5,5 hpf meaning while some of these transcripts will be newly synthesised, the majority of processing observed is necessarily actively modified maternally inherited mRNAs. The release or sequestration of specific mRNAs into granules, depending on their binding types, could also influence the overall mRNA landscape. My data suggest that the early embryonic transcriptome is more complex than previously recognised, with approximately 10000 mRNAs exhibiting 35000 distinct 3' ends, including previously unannotated 3'UTRs.

Longer 3'UTRs provide additional cis-regulatory motifs, which serve as binding sites for RNA-binding proteins and miRNAs. My results suggest that differential 3'UTR usage may contribute to temporal control over mRNA stability and translation. For instance, mRNAs with short 3'UTRs may be preferentially translated before fertilisation but rapidly degraded thereafter, while transcripts with long 3'UTRs persist and are actively translated at later stages by creating protective, stable structures. This supports a model in which developmental transitions are led not just by mRNA synthesis and degradation but also by selective retention of specific 3'UTR isoforms.

Changes in APA have been reported at later stages of zebrafish, typically resulting in the usage of longer 3'UTRs (Ulitsky et al, 2012). These APA events, occurring well after ZGA, likely facilitate the complex regulatory needs of organogenesis and tissue differentiation. However, the APA shifts I describe here, occurring before ZGA, represent a distinct mode of mRNA regulation that precedes widespread zygotic transcription.

I also observed hundreds of mRNAs exhibiting IPA events. Since IPA-generated mRNAs were detected as early as 0 hpf, their apparent lengthening or shortening may result from the selective loss of particular isoforms or a negative feedback activity. While zygotic transcription may contribute to some IPA-associated lengthening events, the overall regulatory mechanisms remain to be fully determined. Given that IPA can lead to the inclusion of alternative last exons, my findings suggest that shifts in IPA usage may play a role in modulating protein isoform expression during early development.

Overall, this chapter revealed the dynamics of 3'UTR processing and identified novel regulatory modes in the post-transcriptional regulation of gene expression during zebrafish development.

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Chapter III

miRNA library and effect of mRNA length on protein translation.

ABSTRACT

In chapter I and II, I discovered the shortening of *pou5f3* mRNA and the shortening and lengthening of thousands of mRNAs in 3 distinct patterns classified as class I, class II and class III. Although, it is still obscure how this processing happens, different patterns could call for different processing pathways. Also, the systemic loss of binding sites from the UTRs of a number of transcripts implies regulatory factors not being able to act on said mRNA, including binding proteins and miRNAs. In animals, miRNA tend to bind through a short sequence called “seed” oftentimes to a target sequence in the 3'UTR of 6 to 8 nucleotides, binding that is multifactorial and impacted by the mRNA’s conformation. In this chapter, I analysed candidate miRNAs by using the readily available database and generating a miRNA library. This custom-built miRNA library demonstrated the accumulation of 100 miRNAs in embryos at 1 hpf and identified 26 previously unknown miRNAs. One of the 4 with the highest confidence score may target the 3' end sequences of *pou5f3* mRNA. In nuclear processing, one of the main actors in mRNA 3' end processing is CPSF6 (Cleavage and Polyadenylation Specificity Factor 6), a key component of the polyadenylation machinery. I found that the amount of Cpsf6 slightly increased by 4 hpf, right after *cpsf6* mRNA was lengthened. This tendency was also observed with *elavl1* which had increased expression after lengthening. These results suggest the potential role of 3'UTR lengthening and the facilitation of 3'UTR processing by synthesised CPSF6.

INTRODUCTION

In the previous chapter, I demonstrated an overwhelming presence of mRNA processing. In the physiological and pathological, changes in the 3'UTRs through nuclear RNA processing are common occurrence, and several causes have been contemplated (Danckwardt et al, 2008, Ji et al, 2009, Thomsen et al, 2010, Li et al, 2025). To understand what could entail mRNA length change, it is useful to research the impact of the shortening or lengthening of processed mRNA throughout the early development. In chapter I, I have shown *pou5f3* shortening to impact its translation when a *pou5f3* relieved of 72 nucleotides appeared to show more translational efficiency than its longer counterpart.

Similarly to developmental cells, there tend to be higher incidence of APA in cancer cells (Masamha et al, 2014). Cancer cells frequently use proximal poly(A) sites, resulting in mRNAs with shorter 3'UTRs. This removes binding sites for miRNAs and RNA-binding proteins, reducing post-transcriptional repression and enhancing oncogene expression (Sun et al, 2024). For instance, MYC oncogene in liver cancer undergoes APA, leading to shorter mRNA forms that escape miRNA repression and boost tumour progression (Venkat et al, 2020). In this regard, the change of length of the 3'UTR impacts the binding of miRNA.

In chapter I, I showed the possibility of micro-RNA (miRNA) binding to *pou5f3* long but not short 3'UTR using a miRNA prediction binding tool. However, miRNA tools usually do not include zebrafish as a model or are obsolete. Many miRNAs remain unannotated.

miRNA are small, non-coding RNAs that play crucial roles in post-transcriptional gene regulation. They typically originate from precursor miRNAs (pre-miRNAs), which form characteristic stem-loop (hairpin) structures. Identifying novel miRNAs, particularly in not so well studied organisms like zebrafish, requires a combination of structural predictions, sequence homology searches, and target validation. Key features of a pre-miRNA include a structure with minimum free energy (MFE) in the range of -30 to -70 kcal/mol. Lower MFE values (< -100 kcal/mol) may indicate a more complex RNA structure, such as a long non-coding RNA (lncRNA), rather than a miRNA precursor (Bonnet et al, 2004). After extracting small RNA and small RNA-sequencing, using computational methods to confirm the existence of novel miRNAs is the most accurate methodology.

miRNAs regulate gene expression primarily through mRNA degradation and translational repression. By mediating mRNA processing through both degradative and non-degradative pathways, miRNAs play a crucial role in fine-tuning gene expression, particularly in developmental and disease contexts (Vasudevan et al, 2012, Martin et al, 2014, Orang et al, 2014, O'Brien et al, 2018, Suzuki, 2023).

To generate miRNA, pre-miRNAs are processed in turn by Drosha (in the nucleus) and Dicer (in the cytoplasm). The study of mutants demonstrated severe developmental delay or even lethality depending on the extend and the protein (Drosha or Dicer) (Kim et al, 2016, Pong et al, 2018). Pre-miRNA are typically in a hairpin structure with the 5' strand generating what is called “the guide”, usually incorporated to the RISC complex leading to the degradation of its target. The “passenger” or “star” is its complementary sequence, on the other side of the hairpin, which until recently has been thought as a

simple by-product but is now thought to be of biological significance (Khvorova et al, 2003, Bartel, 2004, Czech et al, 2011, Medley et al 2020).

In chapter 2, I found that one mRNA that was particularly lengthened from 0 to 2 hpf was *cpsf6.* CPSF6 (Cleavage and Polyadenylation Specificity Factor 6) is an essential component of the cleavage factor complex involved in nuclear 3' end processing of mRNAs (Gruber et al, 2023, Liu et al, 2024). Indeed, the polyadenylation complex is, among others, constituted CstF (cleavage stimulation factor), PAP (polyadenylate polymerase), PABII (polyadenylate binding protein 2), CFI and II (cleavage factor I and II) which englobes members of the CPSF family (Elkon et al, 2013). CPSF knockouts have been studied to be lethal or presenting developmental disorders in human, mice and zebrafish (De Prisco et al, 2023).

In this chapter, I analysed miRNAs binding sites of *pou5f3* in comparison using readily available databases. In addition, I sequenced a 1 hpf small RNA library and quantified known miRNAs. I identified novel miRNAs. For both known and newly found miRNAs, I also analysed mRNAs that could be their biding targets. Finally, I looked at the expression of 3 proteins with a significant role in the early development, namely Dicer1, Cpsf6 and HuR, all of which underwent mRNA processing between 0 hpf and 4 hpf. I subsequently found that not only shortening can activate mRNA translation, but also lengthening, highlighting the complexity of 3'UTR changes and what it entails for the early development.

MATERIALS AND METHODS

miRNA library generation

Fifty eggs at 1 hpf were homogenised with RNA grade extraction buffer (EB) [in the Lexogen Small RNA Library Prep Kit (Lexogen)] containing a concentration of 1/100 proteinase K, for 30 minutes at 37°. For small RNA selection, a SPLIT gel column (Lexogen) was used. Small RNA libraries were prepared using the Lexogen Small RNA Library Prep Kit following the manufacturer's protocol. Briefly, 500 ng of small RNA was used as input, and 3' and 5' adapters were sequentially ligated to small RNAs. Adapter-ligated RNA was subjected to reverse transcription followed by PCR amplification. The final library was size-selected using magnetic bead purification (in kit) to enrich fragments corresponding to mature miRNAs (~140–160 bp, including adapters). Library quality and concentration were assessed using Tape Station (High D1000) (Agilent) before sequencing. High-throughput sequencing was performed on an Illumina Miseq 150SE (Illumina) with single-end 140 bp reads settings.

miRNA analysis

The sequencing data underwent quality control using FastQC to assess read quality, adapter content, and GC distribution. Reads were then trimmed using Fastp to remove adapter sequences and low-quality bases. High-quality reads were aligned to the zebrafish reference genome GRCz11 (Ensembl release 110) using Bowtie. Read quantification was performed with FeatureCounts. RNA structure was assumed by RNAfold. New RNA sequences were inferred using MiRdeep2 (Freeländer et al, 2012) with collapsed file earned using MiRdeep2 mapper. Mature and hairpin structures were

verified using MiRdeep2 quantifier. For reference preprocessed and mature file were downloaded from miRbase, a tool developed by Kozomara and colleagues (Kozomara et al, 2019). For new miRNA sequenced, a binding predicting tool (psRNA target, 2017 version) was used to forecast which miRNA would bind to which 3'UTR. The 3'UTR file was generated from USCS, set on using the Ensembl GRCz11 reference file. GO was performed using ShinyGO (version 0.82).

Dicer antibody generation

Dicer 3' end ORF (1290 nucleotides at the 3'end of the ORF) was amplified by PCR and ligated into pGEX-KG vector at Eco RI/Sal I sites to produce glutathione S-transferase (GST)–tagged proteins. The recombinant proteins were expressed in *Escherichia coli* (XLI) and purified by SDS-PAGE, followed by electroelution in Tris-glycine buffer. The purified proteins were dialysed with 1 mM Hepes (pH 7.5), lyophilised, and then injected thrice (200 ug, 100 ug and 100 ug) into two mice. The obtained antisera were affinity-purified with recombinant GST–Dicer protein electroblotted onto the Immobilon membrane.

Western blotting, IP/RT-PCR

Protein fraction was extracted from a number of zebrafish eggs at different time points with twice 2x amount of extraction buffer (100 mM β-glycerophosphate, 20 mM HEPES, 15 mM MgCl2, 5 mM EGTA, 1 mM dithiothreitol, 100 μM (p-amidinophenyl) methanesulfonyl fluoride, and 3 μg/mL leupeptin; pH 7,5) with 1% Tween 20. After centrifugation at 5000 rpm for 15 min at 4°C, the supernatant was collected. After SDS-PAGE, the gel was blotted to an immobilon membrane for 3 hours. The membrane was

then incubated for 1 hour in a blocking (4% skim milk and 0.1% Tween 20 in TBS) and overnight at room temperature with anti-Dicer (A and B), mIgG or no antibody. For IP/RT-PCR, the protein samples were incubated with anti-Dicer1 antibody and Sepharose beads at 4°C overnight and then ran on SDS gels, after which, blotted on the immobilon membrane. After extracting mRNAs from the beads using TRIzol reagent, RT-PCR was performed. For this, 150 uL out of the 200 uL of EB and beads mix were collected, washed and added to 500 uL of TRIzol. After adding 100 uL of chloroform and centrifuge at 12 000g for 10 minutes, the supernatant was collected, and an equal amount of isopropanol was added. The sample was centrifuged again at 12 000g for 10 minutes and the gathered pellet was washed with 70% EtOH, and then diluted in 50 uL of dDW. 2500 ng of this total RNA sample were used for reverse transcription using Superscript III First-strand synthesis system (Invitrogen) according to the maker's protocol. Then, 2 uL out of the 10 uL of the cDNA were used as an input of PCR using the expand high fidelity PCR system (Roche), following the manufacturer's instructions for a total reaction of 26 uL. The PCR was performed using primer sets specific for *pou5f3* (chap I).

Immunostaining

Zebrafish embryos were fixed in 4% PFA/PBS overnight at 4°C. Following chorion removal, the embryos were incubated for 1 hour in a blocking buffer (4% skim milk and 0.1% Tween 20 in TBS). They were then incubated overnight at room temperature with either an anti-Cpsf6 rabbit antibody (1:50 dilution) (Santa Cruz), an anti-Rpl11 rabbit antibody (1:200 dilution) (Abcam), an anti-Pou5f3 mouse antibody (1:50 dilution) (Sato et al, 2022), an anti-HuR antibody (1:100 dilution) (Santa Cruz) or no first antibody. In

the case of Dicer (anti-Dicer mouse antibody), the dilution was of 1:50 for the first repetition and 1:100 for the 2 second repetitions. The following day, embryos were incubated with an anti-rabbit or anti-mouse IgG Alexa-555 antibody (1:200 dilution) for 1 hour. After mounting, the samples were examined using fluorescence microscopy and an LSM 980 confocal microscope (Carl Zeiss).

RESULTS

Binding site deletion in *pou5f3*

Some prediction tools allow for miRNA binding sites global view. Since miRNA tend to have a short binding sequence of under 10 bases, which also called seed, it is hard to say whether these binding actually happen *in vivo*. However, they give an idea of the impact of deleting a region in a particular transcript. Using TargetScanFish, we have noticed that putative miR-737 binding sequence got deleted in *pou5f3* mRNA when the short isoform was birthed (Fig 6C). The shortening also stops right before another potential seed (miR-135). When looking at the expression of both miRNAs using miRbase, which acts an as-comprehensive-as-it-can-be database, both are found in the ovary. In the case of miR737, both the guide and the passenger can largely be found in the ovary (3p: 19 Reads Per Million (RPM), twice the mean; 5p: 150 RPM) (Fig 15A). In the case of miR-135, miR-135c-5p is the most present with 269 RPM in the ovary (Fig 15B). The other paralogs do not show the same level of expression however, for miR-135a-2-5p, it is higher than the mean.

miRNA expression at 1 hpf

I have created an Illumina compatible miRNA library from a small RNA enriched sample. Illumina sequencing revealed a particularly unbalanced expression with one miRNA having 1457133 reads picked up (Fig 16A upper), another 107348 while the next two in the thousands and the others in the hundreds only. The first one is referenced in Ensembl under si:ch73-389k6.1 (Fig 16A). After 3D simulation of the sequence mapped against si:ch73-389k6.1, the centroid model appeared to resemble that

of a pre-miRNA (Fig 16A lower). The lack of stability given by the minimum free energy of -80 kcal/mol indicates also a possibility for this to be a pre-miRNA.

Surprisingly, at 1 hpf, no member of the miR-430 family, which has been known to be the most abundant miRNA in later stages (Giraldez et al, 2007) could be picked up in my library. On the other hand, dre-miR-21 and 22 targeting genes were broadly expressed. dre-miR-181 was also expressed at 1 hpf. These miRNAs are known to be important for the development (Kumarswamy et al, 2011, Kos et al, 2016, Indrieri et al, 2020, Dehghan et al, 2021). Using TargetScanFish, a list of the miRNAs targets was generated. I reduced the selection to mRNAs expressed in the ovary to focus on the maternal expression landscape. For all of three miRNAs targets, RNAs were coding, longer, especially in the 3'UTR, with on average, a greater number of isoforms (Fig 16B, 17A-B). Since the targets resemble class II mRNAs shown in chapter II, they may be regulated by miR-21, miR-22 and miR-181.

Having a closer look on mRNAs targeted by miR-21, they were involved in negative feedback transcription/translation processes whereas mRNAs target by miR-22 were involved in positive transcription/translation regulation, indicating a fine tuning from their complementary actions. miR-181 targets are mostly acting on localisation of different types of molecules. Overall, all three targets and therefore miR-21, miR-22 and miR-181 showed to be involved in developmental processes, and most likely important actors of the early development of zebrafish.

Unreferenced miRNAs are expressed in zebrafish early-stage embryo

Using MiRdeep2, which allows both the quantification of pre-miRNAs, mature miRNAs and identification of new miRNAs, out of the approximately 100 miRNAs in the library, 26 novel, unreferenced miRNAs were discovered. The majority of them presented both the precursor and the active form at 1 hpf with 5 of them being expressed in significant amounts, hinting toward an active production of miRNAs even in the early stages of zebrafish development. Out of these, 4, with the highest confidence score, were further analysed (Fig 18). psRNATarget, a tool that is able to predict miRNAs target sites from a miRNA's sequence, was used for the 4 new miRNAs. Gene ontology analysis revealed RNA targets were coding, long (in particular in the 3'UTR), with more isoforms than the average, resembling class II mRNAs (Fig 19A-D).
Out of the newly discovered miRNA by miRdeep2, the one with the most confidence score binds to the 72 bases deleted sequence of *pou5f3* (Fig 20).

An active endonuclease, Dicer1

miRNAs being present in both their premature and mature form, their processing could be an active and ongoing event in the early development, which would necessitate the action of the endonuclease Dicer1. An antibody of Dicer1 was created to specifically detect zebrafish Dicer1 (Fig 21 A). After performing immunoprecipitation of Dicer1 at both 0 hpf and 4 hpf, RT-PCR of *pou5f3* showed that Dicer1 did not bind directly to *pou5f3* (Fig 21 B). Another more plausible scenario is seeing Dicer1 acting via miRNAs generation. In the change point analysis, *dicer* mRNA was shortened from 0 hpf to 2 hpf, but not from 2 hpf to 4 hpf, suggesting a potential translational activation quite early on in zebrafish development.

When staining eggs through 0 until 6 hpf, Dicer1 was detected from 0 hpf with an increasing presence. It shows both nuclear and cytoplasmic and minor nuclear expression from 0 hpf onwards (Fig 21 C,D). From 2 hpf, clusters seemed to appear in the shape of bigger aggregates, sometimes covering the entire nucleus, sometimes in a vesicle-like fashion, in the cytoplasm (Fig 21 D).

Two different patterns of expression for mRNA lengthening

Cpsf6, a member of the cleavage and polyadenylation specific factor family, is important for APA by selecting a PAS. It has been predicted, from previous studies, to be involved in 3' end RNA processing (Gruber et al, 2023, Liu et al, 2024). APAlyzer results showed *cpsf6* mRNA to be lengthened through a change of PAS, notably and standing out from others, with a higher relative expression difference, from 0 hpf to 2 hpf. When checking its localisation, it appeared to be not only cytoplasmic (Fig 22 A) - although no expression change was detected overtime (Fig 22 B), but also nuclear from 2 hpf (Fig 22 C) but this tendency increases furthermore from 4 hpf. Interestingly, at 4 hpf especially, only certain mitotic stages of the cell expressed nuclear CPSF6 while others repelled it (Fig 22 D). Elavl1, which is also lengthened from 0 hpf to 4 hpf (Fig 23 A), was expressed continuously at increased levels from 0 hpf to 4 hpf, while consistently forming granular-like condensates in the cytoplasm, although the spatial expression of the protein was the same all throughout 0 hpf to 6 hpf, its expression increased gradually to reach its peak at 4 hpf (Fig 23 B). HuR (Elavl1) itself is known to be involved in granule formation (Brennan et al, 2001, Tsai et al, 2008, Huai et al, 2024). Western blotting confirmed this results with a significant difference between

HuR/Elavl1 expression at 0 hpf compared to 4 hpf (Fig 23 C). Interestingly, from 6 hpf, its concentration decreased as RNA stabilisation becomes less needed.

DISCUSSION

My study revealed that shortening and lengthening of mRNAs are common occurrences observed in zebrafish as early as before the zygotic genome activation even starts. The change of length would mean a change of three-dimensional structure which entails different binding site being accessible, or, on the contrary inaccessible. In the case of shortening, we have studied what could be responsible actors for the mechanism to start. *pou5f3* has two putative miRNA binding sites in their 3'UTR which one being fully deleted in its shorter version from 2 hpf. The RNA configuration changing with its length, accessibility to other sites could also be modified by the shortening of its mRNA. miRNA seeds are relatively short, and several pre-miRNAs can create paralogs. On top of that, it is a well-oiled machine with back up pieces at all points. For instance, the guide miRNAs were thought for a long time to be the only active ones, but prior research has shown that even the passengers have biological impact and can regulate mRNAs themselves (Bang et al, 2014, Gao et al, 2020). In plants, miRNAs are capable of binding nearly perfectly to their target and they have been widely studied and proven to be important for development and creating stress granule by recruiting other proteins, including endonucleases (Dolata et al, 2016). It is thought that miRNA binding does not necessarily lead to mRNA degradation in mammals but only translation repression. Unfortunately, miRNA annotation is widely incomplete for zebrafish. miRNA-sequencing counters this defect but there is need for more in-depth analysis as well as *in vivo* assessment. miR-737 and miR-135 might very well be acting in *pou5f3* shortening but the presence only of the seed is not proof enough since it is so short which means we cannot exclude a simple coincidence. While 5p miRNA tend to be more abundant

and functionally more active, 3p have been proven to have a role of their own and biologically significant.

In general, all miRNAs quantified at 1 hpf, be it the newly discovered ones or the already well-known to act in the development miRNAs seemed to target class II 3'UTR mRNAs. This could mean the processing of mRNAs differ according to their class. One of the newly discovered miRNAs may target *pou5f3* with 2 potential seeds which would locate in the 72 bases-long sequence being deleted from 0 hpf onwards.

One of the hypotheses for the shortening to happen in mRNAs would require an endonuclease. Dicer1 endorses this role among others and expresses from the earliest stages. The shortening of *dicer1* mRNA hinted towards a translational activation which seemed to show in the form of creating complexes from 4 hpf. Its presence in the nucleus is not an isolated example with Dicer have nuclear functions in mice and human, even performing similar role as Drosha (Burger et al, 2015). It has been shown before that in a Drosha deficient model, Dicer would compensate by processing certain RNAs in the nucleus (Kim et al, 2016). Other nuclear roles of Dicer include heterochromatin formation (Fukagawa et al, 2004), dsRNA processing and DNA Damage Repair (DDR) (Vermeulen et al, 2005, Burger et al, 2015, Francia et al, 2016).

In zebrafish like in other species, a CPSF member selects a PAS through a, for instance, AAUAAA motif (Chan et al, 2014, Liu et al, 2024). Some CPSF subunits interact with RNA-binding proteins (RBPs), which can priorities the selection of an alternative PAS, leading to alternative 3' UTR isoforms. CPSF6 levels have also been shown to be correlated with RNA length by influencing on PAS binding (Ge et al, 2024). In zebrafish, although *cpsf6* mRNA was lengthened, suggesting a translational repression, the protein levels were stable and even decrease after 4 hpf. The case of

HuR (Elavl1) is even more counter intuitive with proteins levels that were increased although *elavl1* mRNA was lengthened.

HuR (Elavl1) is a ubiquitously expressed RNA-binding protein of the ELAV-like family, characterized by its ability to bind AU-rich elements (AREs) located in the 3'UTRs of target mRNAs (Brennan et al, 2001, Lebedeva et al, 2011). Through this interaction, HuR contributes to the stabilization and post-transcriptional regulation of numerous transcripts involved in key cellular processes, including cell proliferation, differentiation, and stress response (Mukherjee et al, 2011, Lebedeva et al, 2011, Schultz et al, 2020). In zebrafish, HuR has been reported to play essential roles during embryogenesis, particularly in regulating mRNAs associated with cell cycle progression and morphogenetic events, thereby ensuring proper development (Chi et al, 2011, Osma-Garcia et al, 2021). After mRNA lengthening, HuR was expressed profusely, indicating the biological significance of mRNA lengthening in certain cases.

GENERAL DISCUSSION

mRNA translational regulation is a vastly studied topic, even in the context of the maternal to zygotic transition. In zebrafish, the common theory being that maternal transcripts persist until clearance from the zygotic genome activation where slowly, newly formed mRNAs take over. Recent studies have challenged this premise (Takada et al, 2023; Bhat et al, 2023, Fishman et al, 2024) with over 90 % of transcripts being maternal at the MZT, although it would depend on the cell type.

I have showed not just one, but thousands of mRNAs are shortened from the earliest stages (including before the ZGA starts, from 0 hpf to 2 hpf). This applied to transcripts encoding necessary transcription factors of the MZT such as Pou5f3, affecting their translation (Fig 20). mRNA shortening would be mechanism that impacts its translation through multiple ways. One of them is binding sites accessibility such as regulatory elements in the 3' UTR that interact with miRNAs or RNA-binding proteins. The loss of hundreds of nucleotides exposes binding sites that were previously masked by RNA secondary structure or occluded by competing factors. For example, in previous studies, HuR (Elavl1), which binds to AREs, who themselves tend to localise in the earlier part of the 3'UTR, have an easier access to the transcripts when the structures formed by a longer 3'UTR are removed. HuR binding allow for the target transcript's stabilisation and even positively regulates translational activation. This happens with p53 or cyclooxygenase 2 in human and mice (de Moraes et al, 2007).

In a similar way to how Adenomatous Polyposis Coli (APC) loss-of-function mutations influence 3'UTR lengthening in human cancers (Gabel et al, 2024, Wang et al, 2024), my research on zebrafish aims to explore how changes in poly(A) site

selection may affect gene expression, particularly in pathways critical for development. For example, in human cancers, alterations in APC expression and its effect on 3'UTR lengthening can influence growth-promoting pathways, such as WNT signalling. Similarly, understanding how RNA-binding proteins like APC in zebrafish affect 3'UTR dynamics could provide insight into the regulatory mechanisms underlying developmental processes and disease. By examining these patterns in zebrafish, we can uncover potential parallels in the regulation of growth and differentiation.

In mice, hnRNP A1 binds to a 71-nucleotide stretch in the 3' UTR of CYP2A5 mRNA, which helps stabilise the transcript and boosts CYP2A5 expression. When this binding site is deleted, the hnRNP A1-dependent regulation is lost and the translation activation is lost (Glisovic et al, 2003). This is an example of the how, on the other hand, lengthening of mRNAs can have the very same effect by a seemingly opposite mRNA regulation mechanism.

Now looking at the processing itself, isoform change is often related to PAS use change. Proximal or distal PAS selection is related to CPSF protein which bind directly to the signal and influence thereby the size of its transcript (Fig 20). CPSF6 is known to favour proximal PAS and therefore, a depletion of CPSF6 has been shown to be directly responsible for a general shortening of mRNAs (Tan et al, 2021, Ge et al, 2024). The corollary is also true, where CPSF6 expression augmentation is linked to mRNA lengthening (Liu et al, 2023), and opposite results when other CPSF members are overexpressed. CPSF6 binds to common PAS and therefore regulates APA sites within the 3'UTR, influencing mRNA stability, localisation, and translational efficiency. By preferentially promoting the use of distal polyadenylation sites, CPSF6 contributes to the production of longer 3'UTRs, which often harbour regulatory elements such as

microRNA-binding sites and RNA-binding protein motifs (De Prisco et al, 2023, Ge et al, 2024). This is critical particularly in developmental contexts, where mRNA length influences gene expression patterns (Chiaromonte et al, 2003, Fansler et al, 2024). On the other hand, reduced CPSF6 activity can lead to proximal polyadenylation site selection, generating shorter mRNA isoforms. This dynamic regulation allows cells to regulate gene expression in response to developmental cues. We have shown *cpsf6* to lengthen early on, before 2 hpf (chapter II, APAlyzer) and its expression to be seemingly a little stronger at 4 hpf both in the cytoplasm and nucleus. At the same time, general APA lengthening becomes stronger from 2 to 4 hpf. Cpsf6 is likely to be involved in mRNA UTR isoform change.

My study raises another question: with the change of 3'end, how does this impact polyA tail length? In line with mRNA translational activation, polyA tail length and 3'UTR length would influence the binding of the PABP family. In zebrafish, like in other organisms, PABPs help regulate mRNA lifespan by interacting with RNA-binding proteins and translation initiation factors (Le et al, 1997, Wigington et al, 2014, Kajjo et al, 2022). These interactions ensure that mRNAs are properly processed and translated, which is essential when zebrafish undergoes transcriptome changes in early development.

Another impact of mRNA processing is the loss of binding sites for miRNAs. The most known mechanism of miRNA's mRNA shortening is silencing by binding the mRNA's poly(A) tail and by deadenylation, reducing mRNA stability and accelerating degradation (Wahid et al, 2010, Dalmay, 2013). In addition to this, recent studies illustrated miRNAs as shortening mediators without complete decay in the nucleus. In some cases, miRNA binding results in an event that generates shorter mRNAs isoform.

These transcripts may retain translational activity, but exhibit altered stability or localisation, affecting their functional output (Xu et al, 2016, Malka et al, 2017). This fine tuning is greatly impacted by their paired binding sites. New potential miRNAs have been identified in an effort to better understand how they modulate gene expression by binding to target mRNAs. Candidates for *pou5f3* include miR-737 or miR-135 or unannotated candidate1 (chromosome 4), with an overwhelmingly strong expression at 1 hpf (over 1 000 000 reads) and candidate2 (chromosome 17) as well as newly identified miRNA1. These findings could be completed by knocking down miRNA candidates and investigate the effects of it at a molecular level by RNA sequencing. *In situ* hybridisation of miR-21, miR-22, miR-181 or herein described novel miRNAs with class II mRNAs could also give stronger proof of miRNAs' involvement. We also found poly(A) tail to be lengthened at later stages in *pou5f3* and *wtn8a*. Whether in this scenario a change of PAS is correlated to poly(A) tail length change during the first few hours of the development could also be. All in all, it could hint towards potentially different types of necessary regulation for mRNAs at a time when timing is of the essence. Taken together, these findings show that mRNAs are processed in a variety of manners, affecting the translation (Fig 20).

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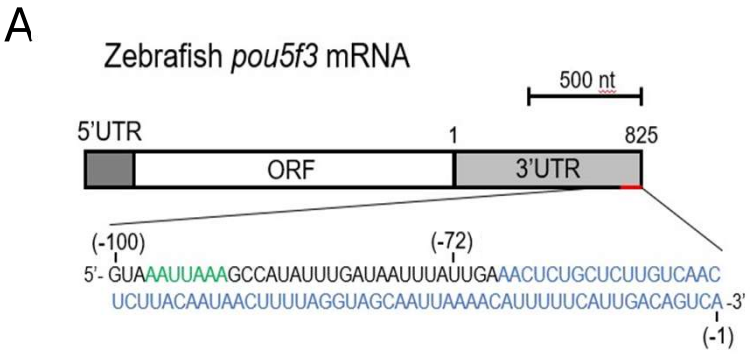
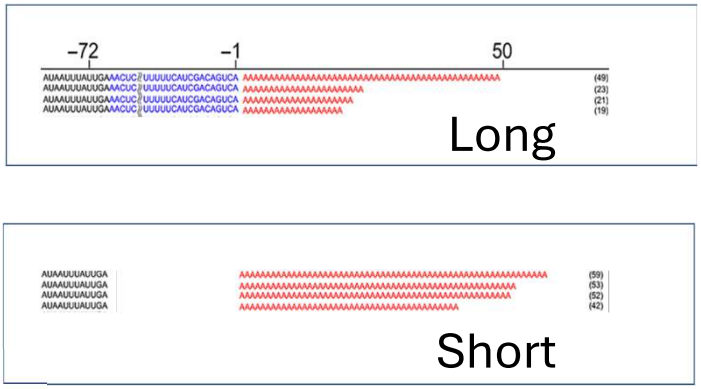
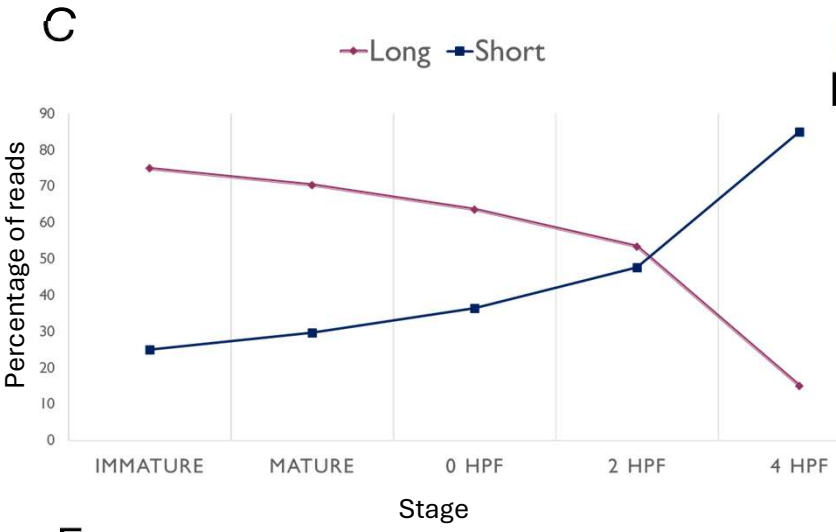
Figures

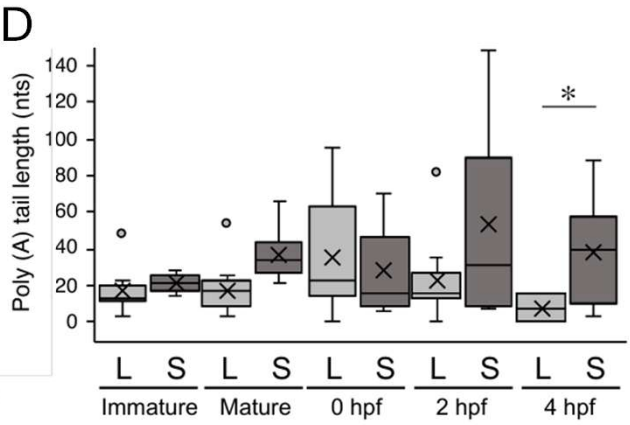
Figure 1

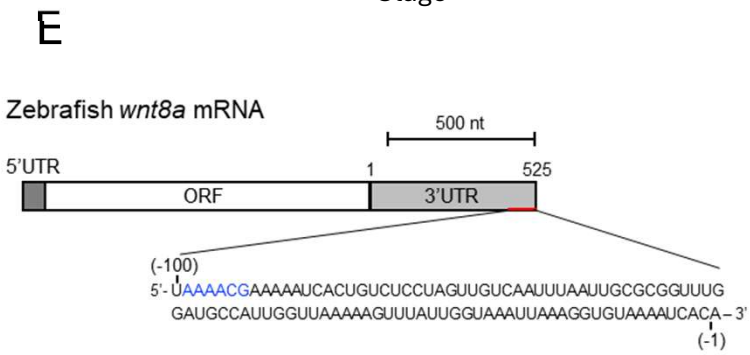
Shortening of *pou5f3* and *wnt8*a from 0 to 4 hpf. (A) 3'UTR sequence encountered for the longest transcript of *pou5f3*. In green, the PAS that was likely used can be observed. The deleted sequence can be seen in blue (B) Examples of PAT assay found *pou5f3* different types of transcripts after sequencing. The longest mRNA had 72 nucleotides more than the shortest one. Other forms were sequenced with a 3'UTR going from +1 to +71 nucleotides compared with the shortest form. (C) PAT assay repartition of *pou5f3* 3'UTR types during each development stage. At first, the dominating type is "long" 3'UTR but the "short 3'UTR slowly gains presence even before the ZGA. At 4 hpf, almost 90% of the transcripts are short. (D) Poly(A) tail length of *pou5f3* during each development stage. When *pou5f3* shortens, its polyA tail lengthen concomitantly. (mean ± SD). **$p < 0.01$, Tukey's multiple comparison test. (E) 3'UTR sequence encountered for the longest transcript of *wnt8a* (F) PAT assay repartition of *wnt8a* 3'UTR types during each development stage. When oocytes are immature, only long transcripts are found. This changes from when occytes gain maturity and at 4 hpf, all transcripts are short. Analogously to *pou5f3*'s way, the transcripts are shortened within the first stages of development. (G) Poly(A) tail length of *wnt8a* during each development stage. The tail seemingly shortens to significantly lengthen at 4 hpf. (mean ± SD). **$p < 0.01$, Tukey's multiple comparison test.

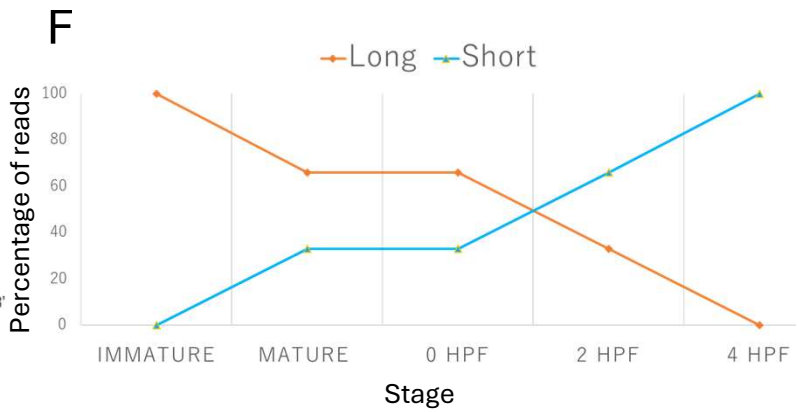

A
Zebrafish pou5f3 mRNA
500 nt
5'UTR
1
825
ORF
3'UTR
(-100)
(-72)
5'- GUAAAUUAAAGCCAUAUUUGAUAAUUUAUUGAAACUCUGCUCUUGUCAAC
UCUUACAAUAACUUUUAGGUAGCAAUUAAAACAUUUUUCAUUGACAGUCA -3'
(-1)

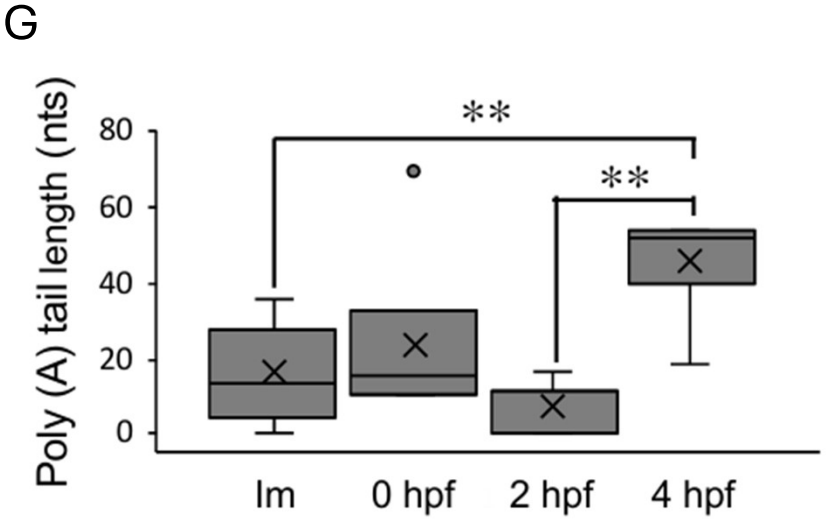

B
−72
−1
50
Long
Short


C
Long
Short
Percentage of reads
IMMATURE
MATURE
0 HPF
2 HPF
4 HPF
Stage


D
Poly (A) tail length (nts)
L S L S L S L S L S
Immature
Mature
0 hpf
2 hpf
4 hpf


E
Zebrafish wnt8a mRNA
500 nt
5'UTR
1
525
ORF
3'UTR
(-100)
5'- UAAAACGAAAAAUCACUGUCUCCUAGUUGUCAAUUUAAUUGCGCGGUUUG
GAUGCCAUUGGUUAAAAAGUUUAUUGGUAAAUUAAAGGUGUAAAAUCACA – 3'
(-1)


F
Long
Short
Percentage of reads
IMMATURE
MATURE
0 HPF
2 HPF
4 HPF
Stage


G
Poly (A) tail length (nts)
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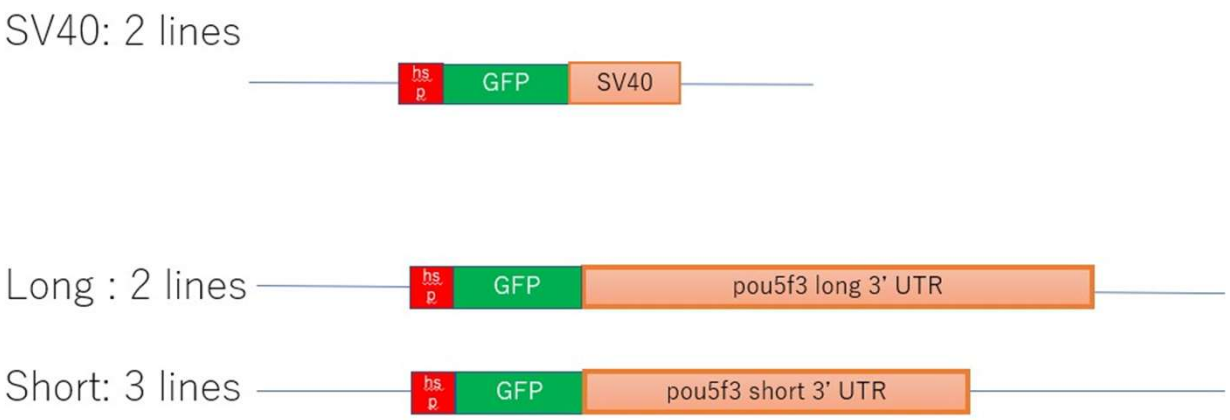
Im
0 hpf
2 hpf
4 hpf

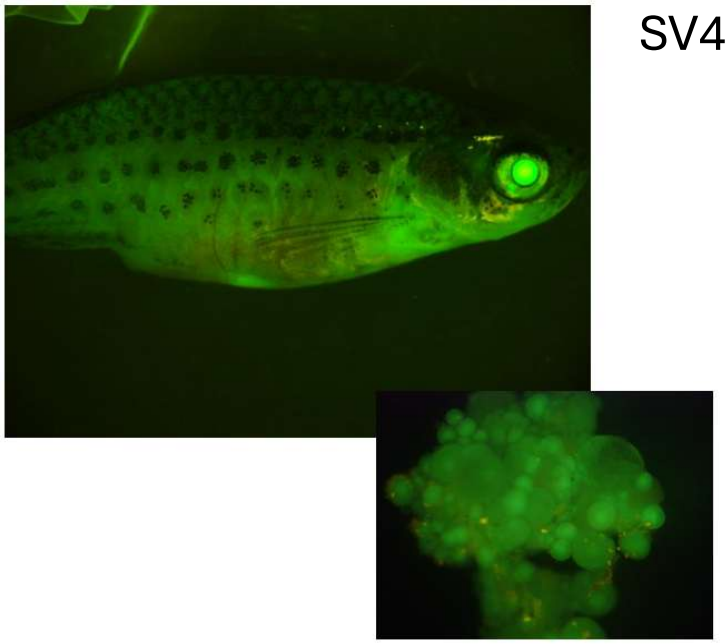
Figure 2

3'UTR transgenic zebrafish. (A) Different types of constructs used. A heat shock promoter and GFP sequence can be found upstream of the 3'UTR of interest (long/short *pou5f3* or SV40) (B) Transgenic zebrafish under fluorescent miscroscope after being heat shocked for one hour. Green fluorescent can be observed all over the body and notably in the oocyte as a result of GFP expression making it an intuitive model for *pou5f3* expression study. (C) Transgenic zebrafish embryo (4-cell stage) under fluorescent light after heat shock at 37°C for one hour. Transgenic eggs can easily be differentiated from non-transgenic eggs. (D) Whole mount in situ hybridisation on the ovary of a fish (TSA system) using GFP probes.

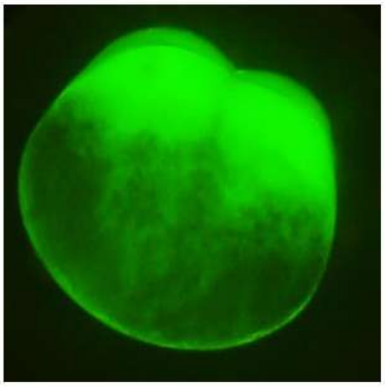
A


SV40: 2 lines
hs p
GFP
SV40
Long : 2 lines
hs p
GFP
pou5f3 long 3' UTR
Short: 3 lines
hs p
GFP
pou5f3 short 3' UTR

B


SV40

C


SV40

D

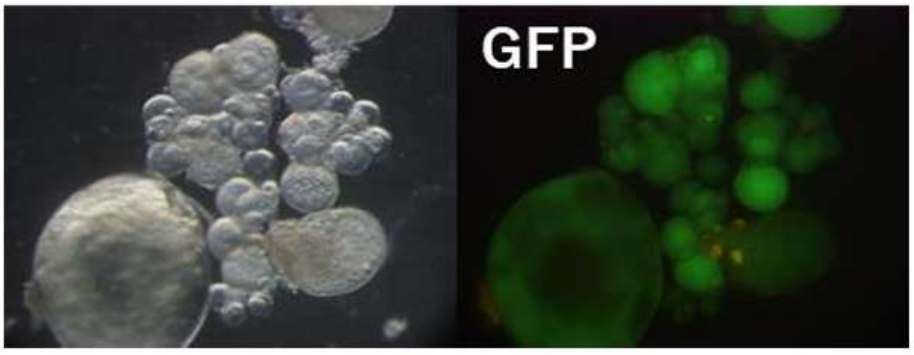
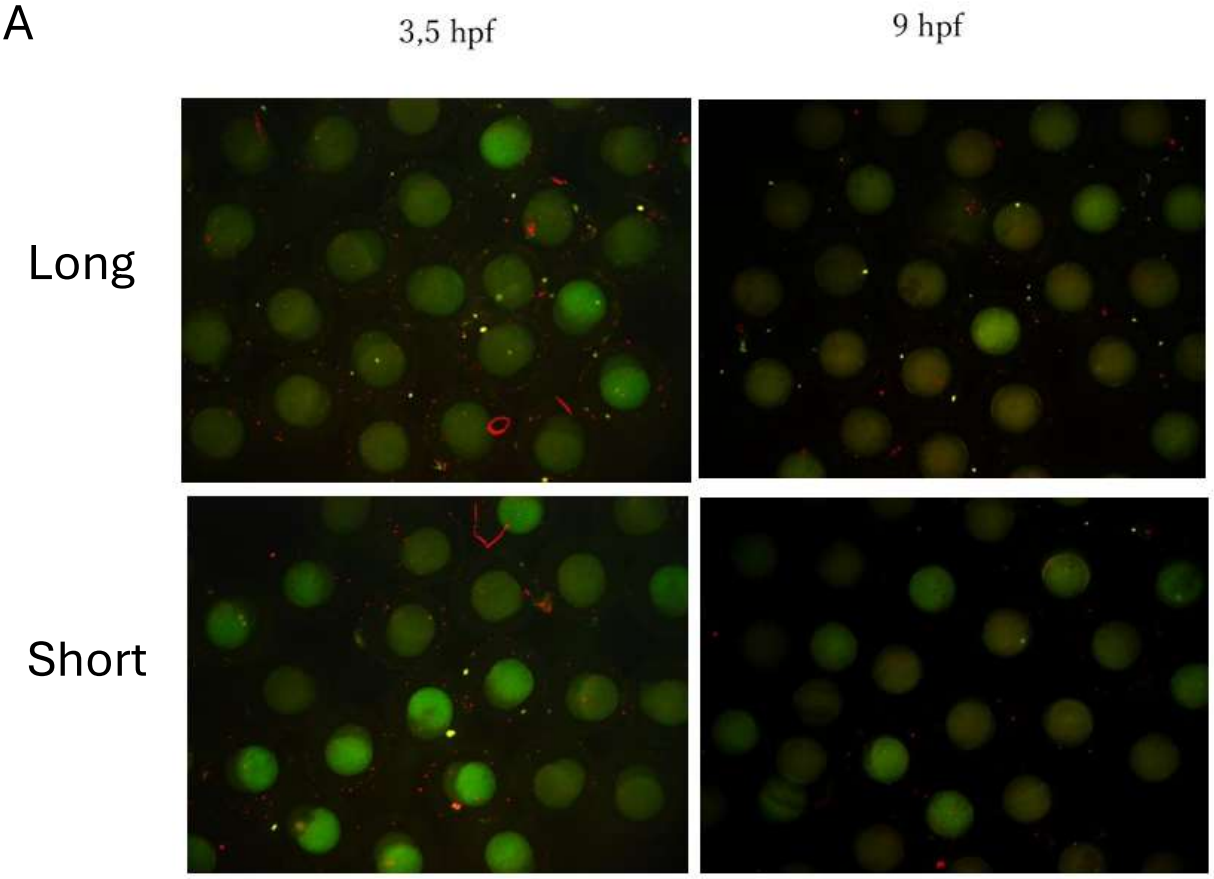

GFP
SV40

Figure 3

GFP expression in short transgenic lines versus long transgenic lines observed under fluorescent microscope. (A) Picture of a selection of eggs at 3,5 hpf vs 9 hpf. Short transgenics appeared to exhibit green fluorescence more often than long ones. (B) Picture of a representative egg at 3,5 hpf vs 9 hpf. The egg from the short transgenic line appeared to be translated more efficiently in the early stages.

A



B

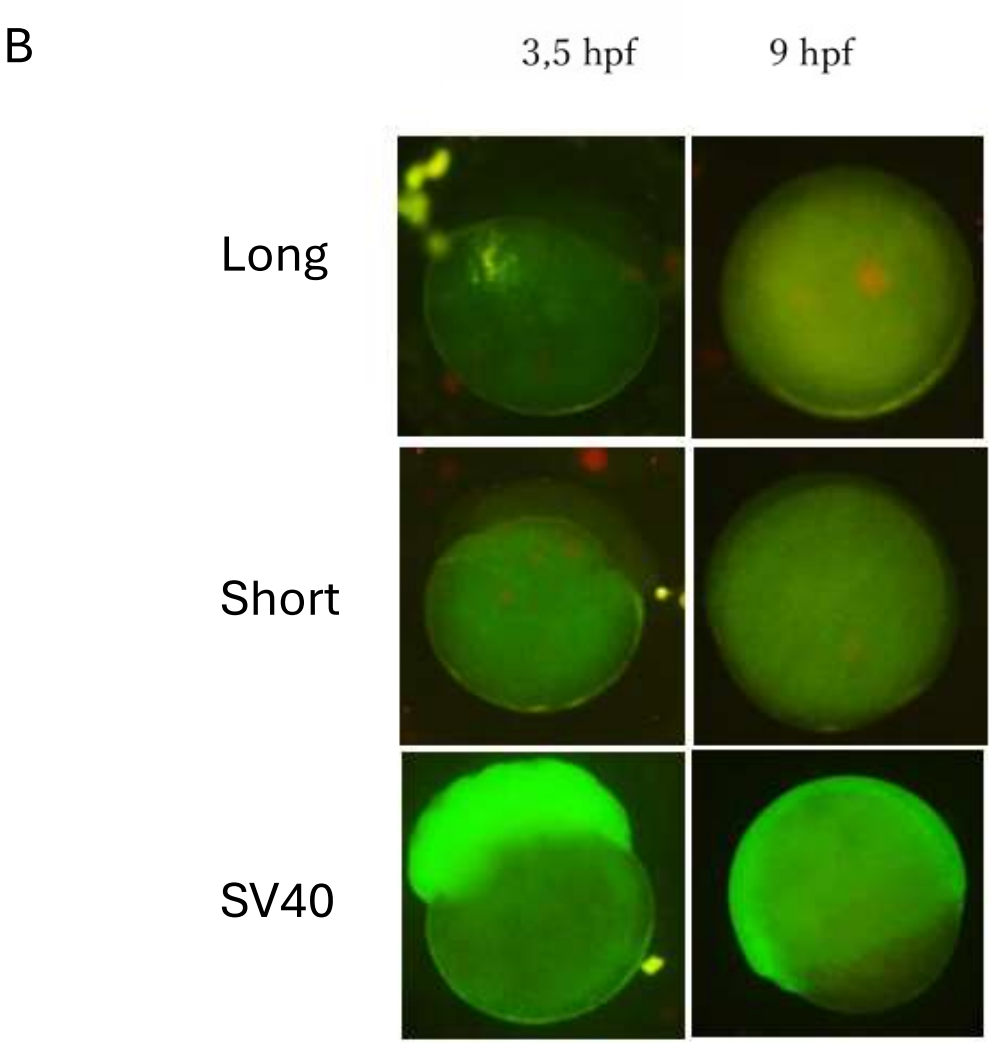
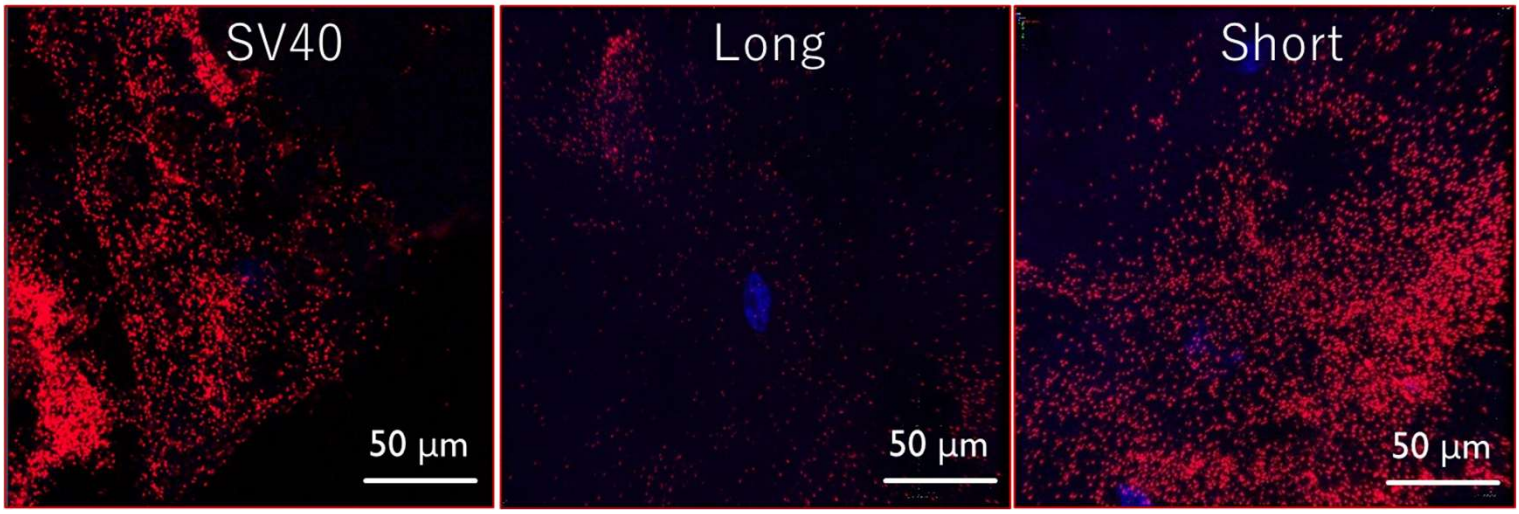


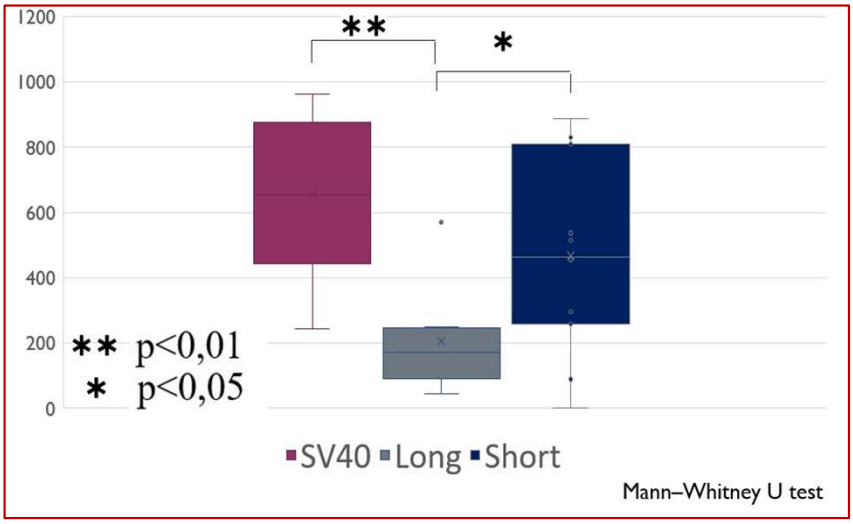
Figure 4

GFP translation efficiency in transgenic lines. (A) GFP translation sites at 2 hpf observed under confocal microscope after puro-PLA. Translation sites can be seen in red, DAPI coloration was used for the nucleus. (B) Translation sites were counted using Image J for 5 to 11 different locations of the samples used. Mann Whitney U test was used for statistics. (C) GFP translation sites at 0,75 hpf observed under confocal microscope after puro-PLA. Short translation site number is significantly higher than long.

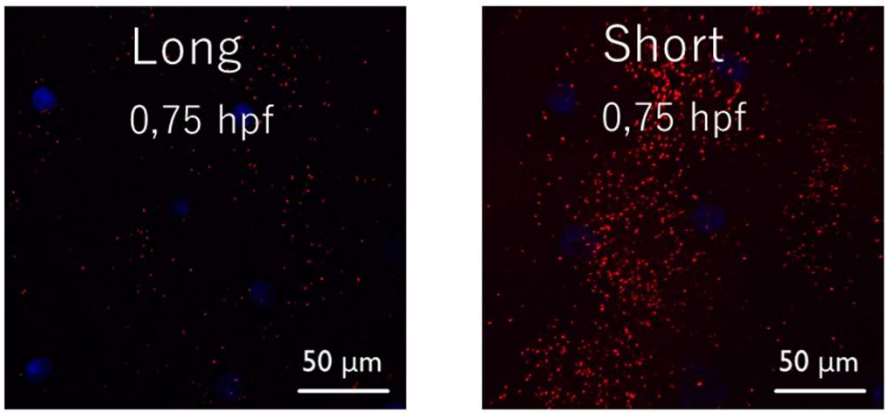
A


SV40
50 µm
Long
50 µm
Short
50 µm

B


1200
1000
800
600
400
200
0
**
*
** p<0,01
* p<0,05
SV40 Long Short
Mann–Whitney U test

C


Long
0,75 hpf
50 µm
Short
0,75 hpf
50 µm

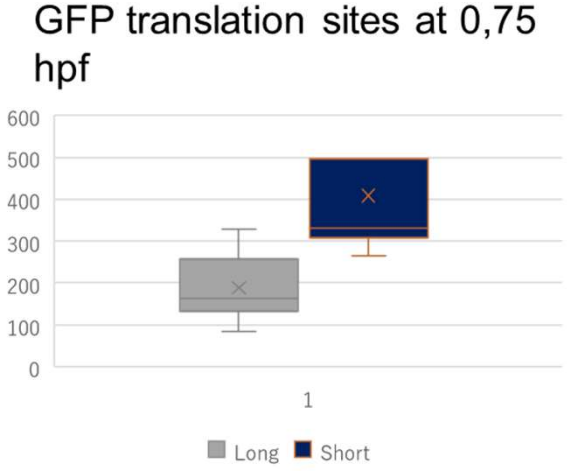
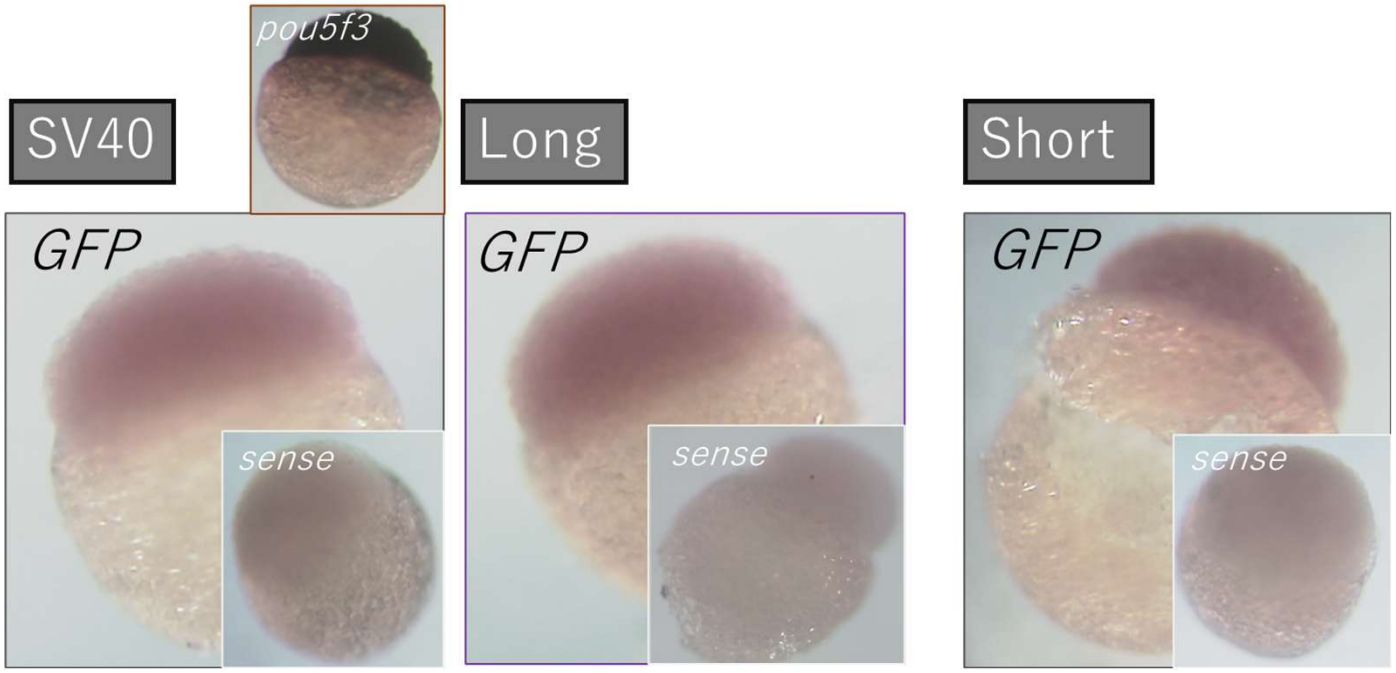

GFP translation sites at 0,75 hpf
600
500
400
300
200
100
0
1
Long
Short

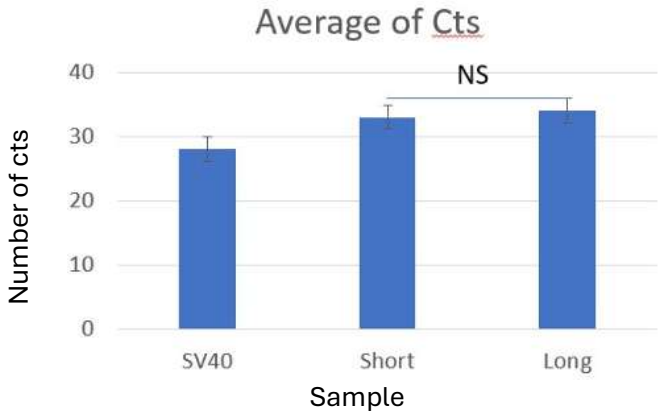
Figure 5

mRNA expression of the reporter (GFP) throughout different development stages. (A*) in situ* hybridisation of GFP of eggs fixed at 2 hpf. Alkaline phosphatase system was used. The same level of coloration could be observed for all embryos, in the animal pole. (B) qPCR for GFP on SV40, short and long transgenic lines. Taking into account the standard deviation, the number of Ct is the same for short and long lines indicating a similar amount of GFP mRNA measured. A similar qPCR was performed for *pou5f3* to show that GFP mRNA quantity correlated (not shown). (C) Whole mount *in situ* hybridisation for GFP of eggs of transgenic or wild-type mother, fixed at 27 hpf. The colouration is brighter for pure-blooded transgenic lines, highlighting the maternal contribution for *pou5f3*.

A


pou5f3
SV40
Long
Short
GFP
GFP
GFP
sense
sense
sense

B


Average of Cts
NS
40
30
20
10
0
Number of cts
SV40
Short
Long
Sample

C

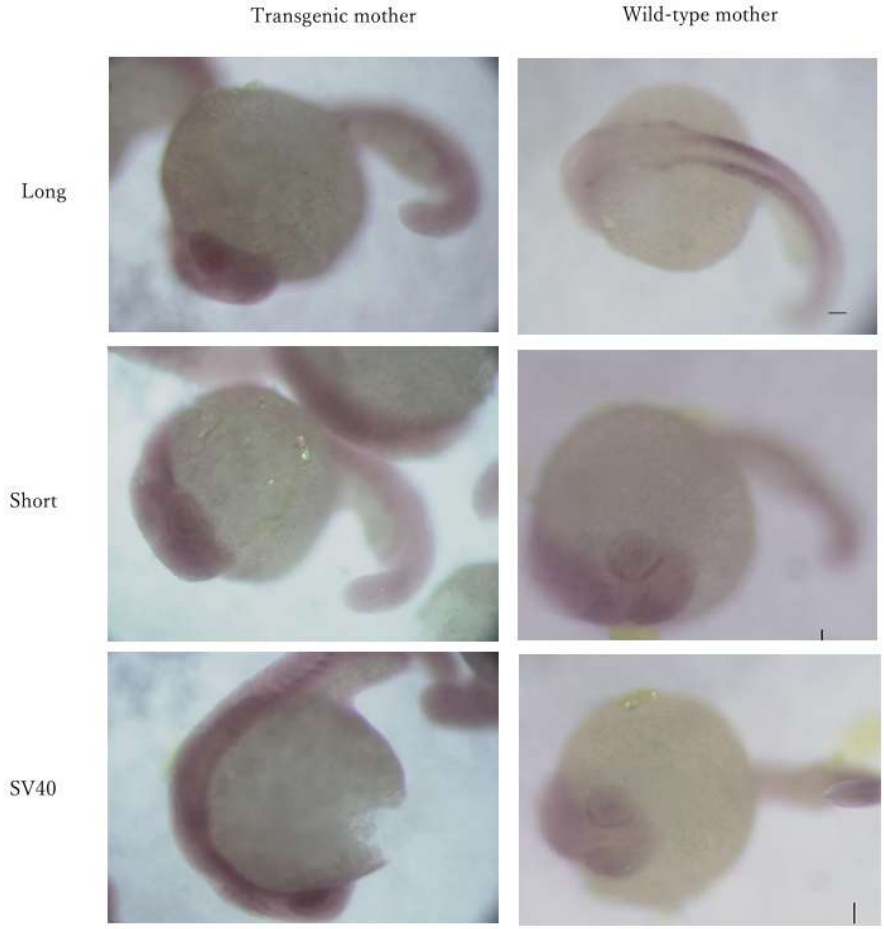
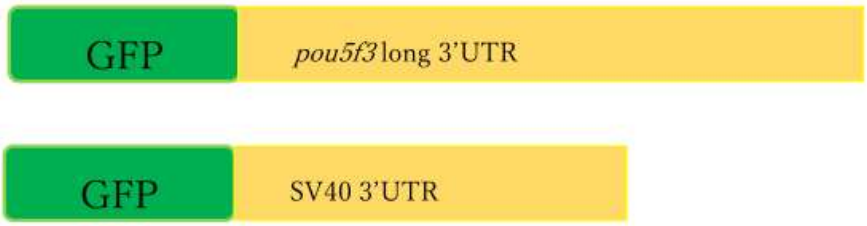

Transgenic mother
Wild-type mother
Long
Short
SV40

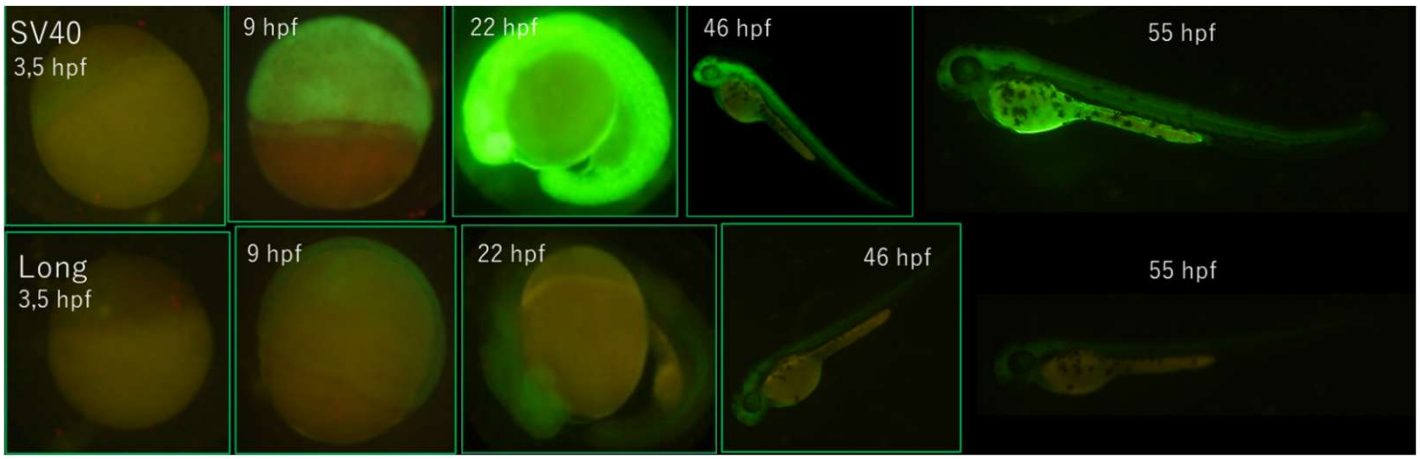
Figure 6

Reporter assay for pou5f3 long 3'UTR. (A) Schematisation of the constructs made for the experiment. GFP is located upstream of *pou5f3* long 3'UTR or SV40 3'UTR to compare their impact on translational activity. (B) *pou5f3* long 3'UTR mRNA reporter assay. GFP expression is much stronger all throughout the stages of early development of SV40 UTR injected fish. (C) miRNA binding predicting tool found one miRNA binding site for *pou5f3* that disappears in the shorter UTR form highlighting the potential impact of shaving off 72 nucleotides.

A


pou5f3 3'UTR
GFP
pou5f3 long 3'UTR
GFP
SV40 3'UTR

B


SV40
3,5 hpf
9 hpf
22 hpf
46 hpf
55 hpf
Long
3,5 hpf
9 hpf
22 hpf
46 hpf
55 hpf

C

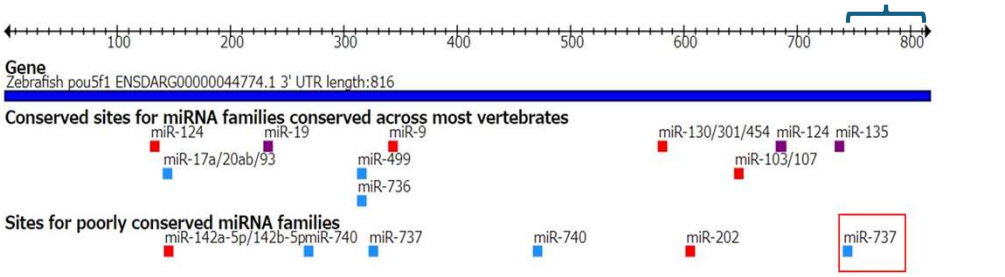
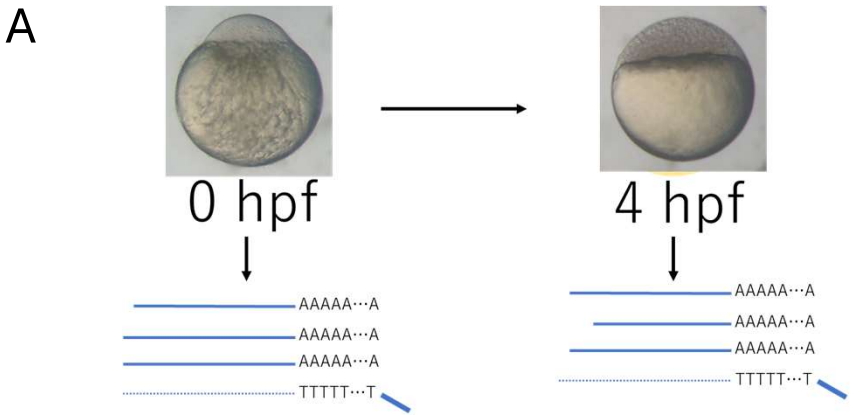
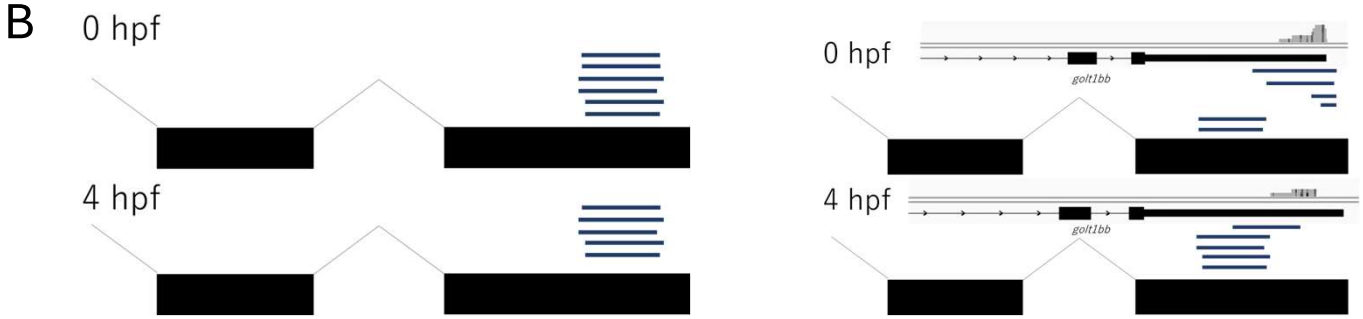

Shortened sequence
100
200
300
400
500
600
700
800
Gene
Zebrafish pou5f1 ENSDARG00000044774.1 3' UTR length:816
Conserved sites for miRNA families conserved across most vertebrates
miR-124
miR-19
miR-9
miR-130/301/454
miR-124
miR-135
miR-17a/20ab/93
miR-499
miR-103/107
miR-736
Sites for poorly conserved miRNA families
miR-142a-5p/142b-5p
miR-740
miR-737
miR-740
miR-202
miR-737

Figure 7

3'UTR-end mRNA sequencing of zebrafish early development. (A) Schema of used RNA-seq technique libraries were made from 0 and 4 hpf samples, using dTTT primers and random primers. Reads with an illumina tag (line in blue, bold) were subsequently sequenced. (B) Schema of read mapping visualised using IGV. A shortening of certain genes could be seen from 4 hpf (right). Here a representative example (golt1bb) is exhibited. Exons are represented by a black box, lines represent an intron. (C) 3 classes of shortening were identified. Class I were genes shortened of less than 150 bases between two stages. Class II were genes shortened of over 150 bases, but still within the annotated 3'UTR. Class III, albeit less common, were shortening to an intron or within the CDS.


A
0 hpf
4 hpf
AAAAA···A
AAAAA···A
AAAAA···A
TTTTT···T
AAAAA···A
AAAAA···A
AAAAA···A
TTTTT···T


B
0 hpf
4 hpf
0 hpf
4 hpf
golt1bb
golt1bb

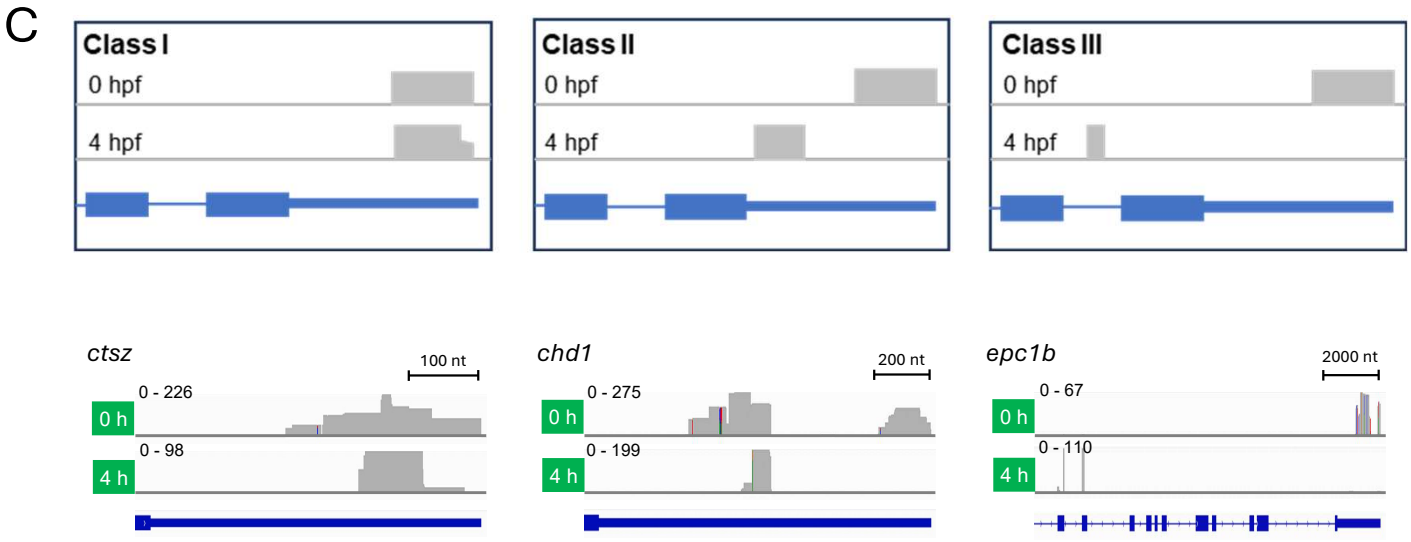
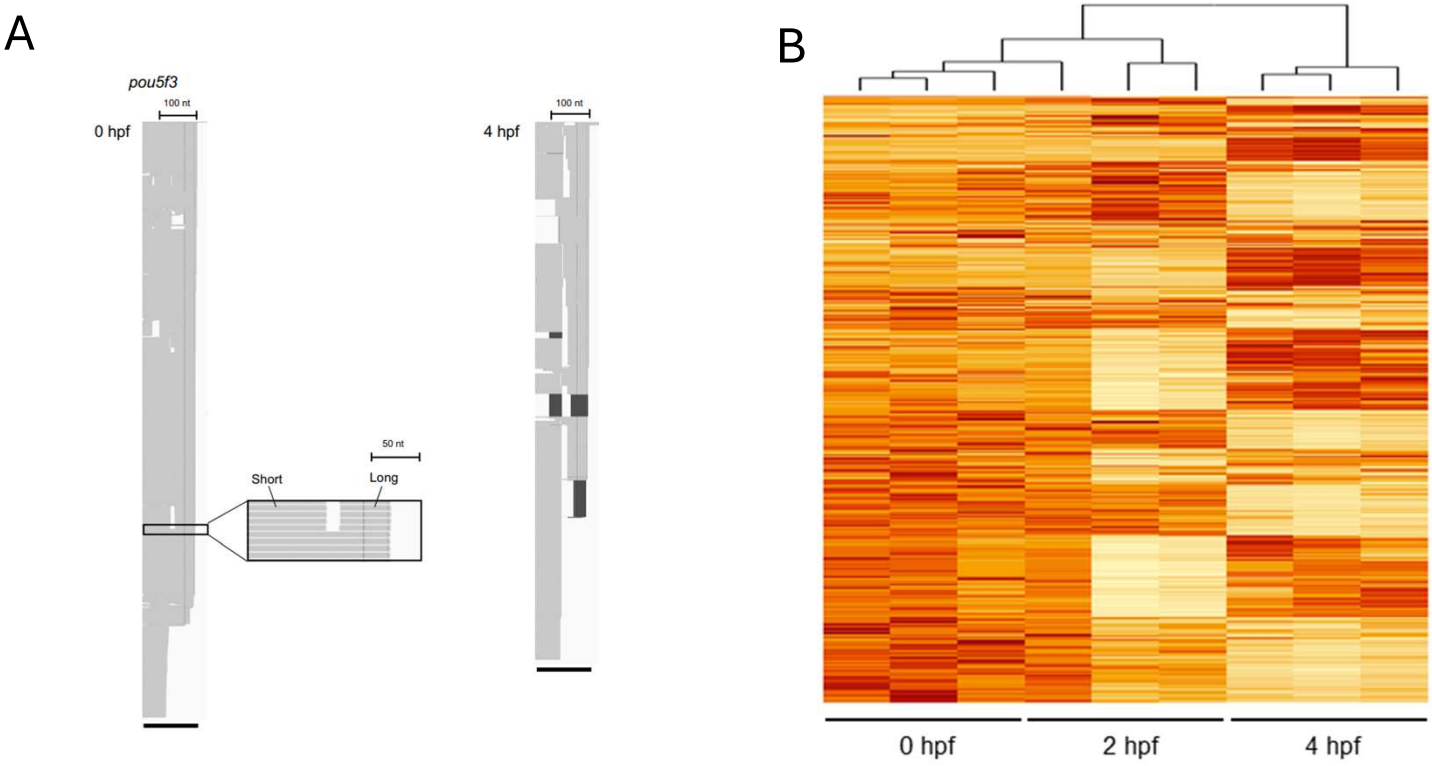
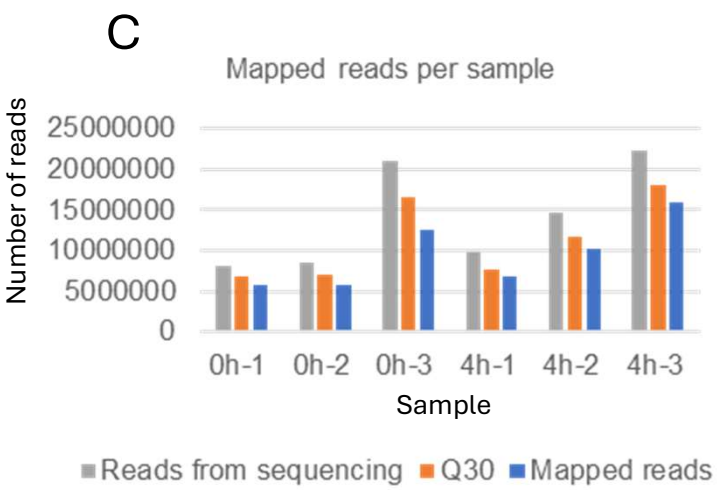

C
Class I
0 hpf
4 hpf
Class II
0 hpf
4 hpf
Class III
0 hpf
4 hpf
ctsz
100 nt
0 - 226
0 h
0 - 98
4 h
chd1
200 nt
0 - 275
0 h
0 - 199
4 h
epc1b
2000 nt
0 - 67
0 h
0 - 110
4 h

Figure 8

Mapping results of RNA-seq. (A) Representation of the issue of using a visualiser coded for regular RNA-sequencing: the track is not accurately depicting the shortening for some genes. Since reads of short transcripts and reads of long transcripts overlap, it shows-up as a non-existent, continuous long read in the track. (B) Heatmap of randomised fpm for each sample. (C) Number of reads, Q30 and mapped reads for each sample. Herein this manuscript, similar sets were chosen for comparison (e.g. 0-1 vs 4-1 or 0-3 vs 4-3). (D) 2 hpf libraries were generated to get a more detailed view in to when the isoform changes started.


A
pou5f3
0 hpf
100 nt
4 hpf
100 nt
50 nt
Short
Long
B
0 hpf
2 hpf
4 hpf

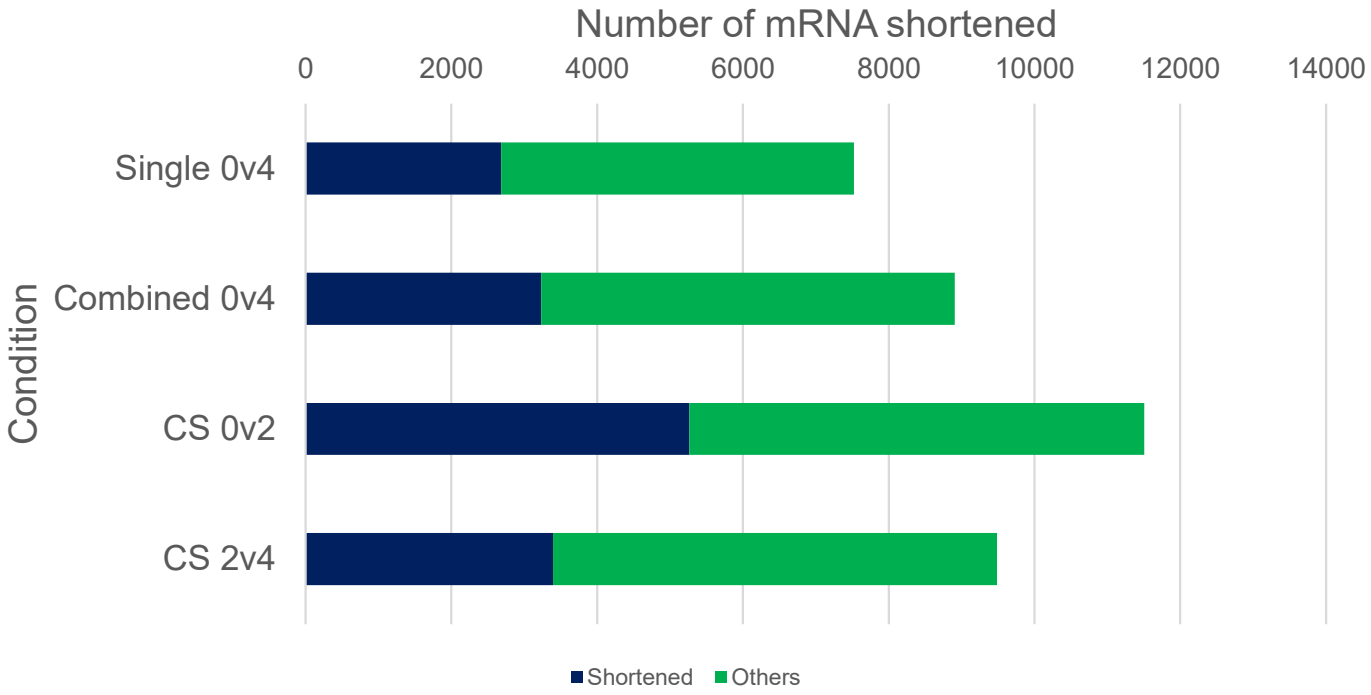

C
Mapped reads per sample
Number of reads
25000000
20000000
15000000
10000000
5000000
0
0h-1
0h-2
0h-3
4h-1
4h-2
4h-3
Sample
Reads from sequencing
Q30
Mapped reads


D
Mapped reads per sample
Number of reads
100000000
80000000
60000000
40000000
20000000
0
2h-1
2h-2
2h-3
Sample
Reads from sequencing
Q30
Mapped reads

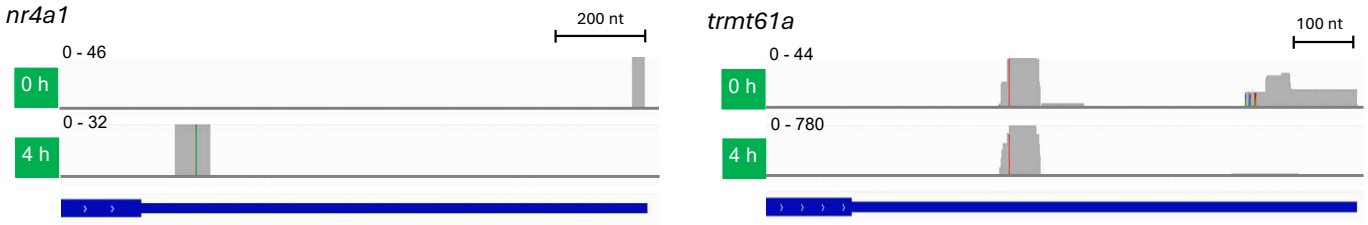
Figure 9

Shortening detected from 0 hpf until 4 hpf. (A) Change point detected shortening for over 10000 genes. Samples analysed were, in order, one single sample of 0 hpf vs one single sample of 4 hpf, a merge of all 3 0 hpf samples vs a merge of all 4 hpf samples, a merge of all 0 hpf samples vs a single 2 hpf sample and a merge of all 4 hpf samples vs a single 2 hpf sample. CS0v2: Combined 0 hpf vs simple 2 hpf. CS2v4: Combined 4 hpf vs simple 2 hpf file. (B) Examples of class II shortening. In some cases, only the shorter transcript remain at the later stage. For other genes, both co-exist at the beginning, and one type disappears later on. (C) Venn diagram of shortened genes from 0 hpf to 2 hpf compared with shortened genes from 2 hpf to 4 hpf. The overlap in olive-green are genes (1269) that both shortened from 0 hpf to 2 hpf and from 2 hpf to 4 hpf.

A


Number of mRNA shortened
0
2000
4000
6000
8000
10000
12000
14000
Condition
Single 0v4
Combined 0v4
CS 0v2
CS 2v4
Shortened
Others

B


nr4a1
200 nt
0 - 46
0 h
0 - 32
4 h
trmt61a
100 nt
0 - 44
0 h
0 - 780
4 h

C

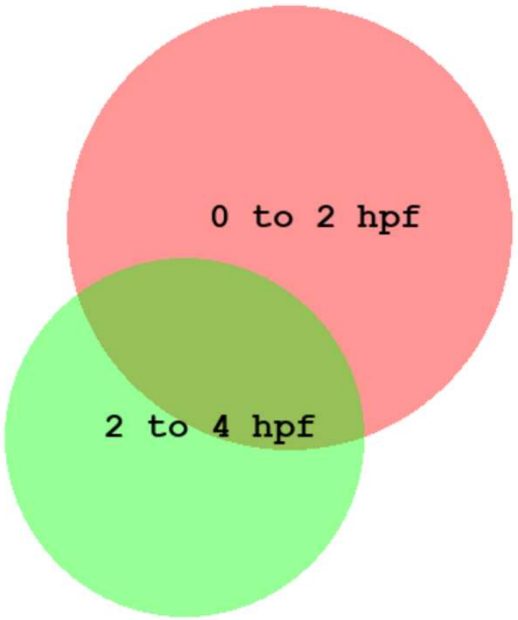

0 to 2 hpf
2 to 4 hpf

Figure 10

RNA processing with a focus on APA at 0 hpf vs 2 hpf. (A) Volcano plot of lengthened (in red) and shortened (in blue) genes. RED = Relative Expression Difference. (B) Box plot of APA change. Numbers represent the number of genes with a significant RED between the two stages. DN = Shortened; NC = Unchanged; UP = Lengthened. (C) (D) Volcano plot and box plot of genes switching from a 3'UTR PAS to an intronic PAS (UP) or the opposite (DN). NC = Unchanged. (E) Violin plot of APA expression. RNA processing is not widespread but exists. (F) Cumulative distribution function of both 3'UTR only processing vs IPA use. IPA use tends to follow 3'UTR processing (G) Examples of lengthening from 0 hpf to 2 hpf (*jupa*) and shortening (*hmga1a*) as well as PAS (red arrowheads) used for each isoform.

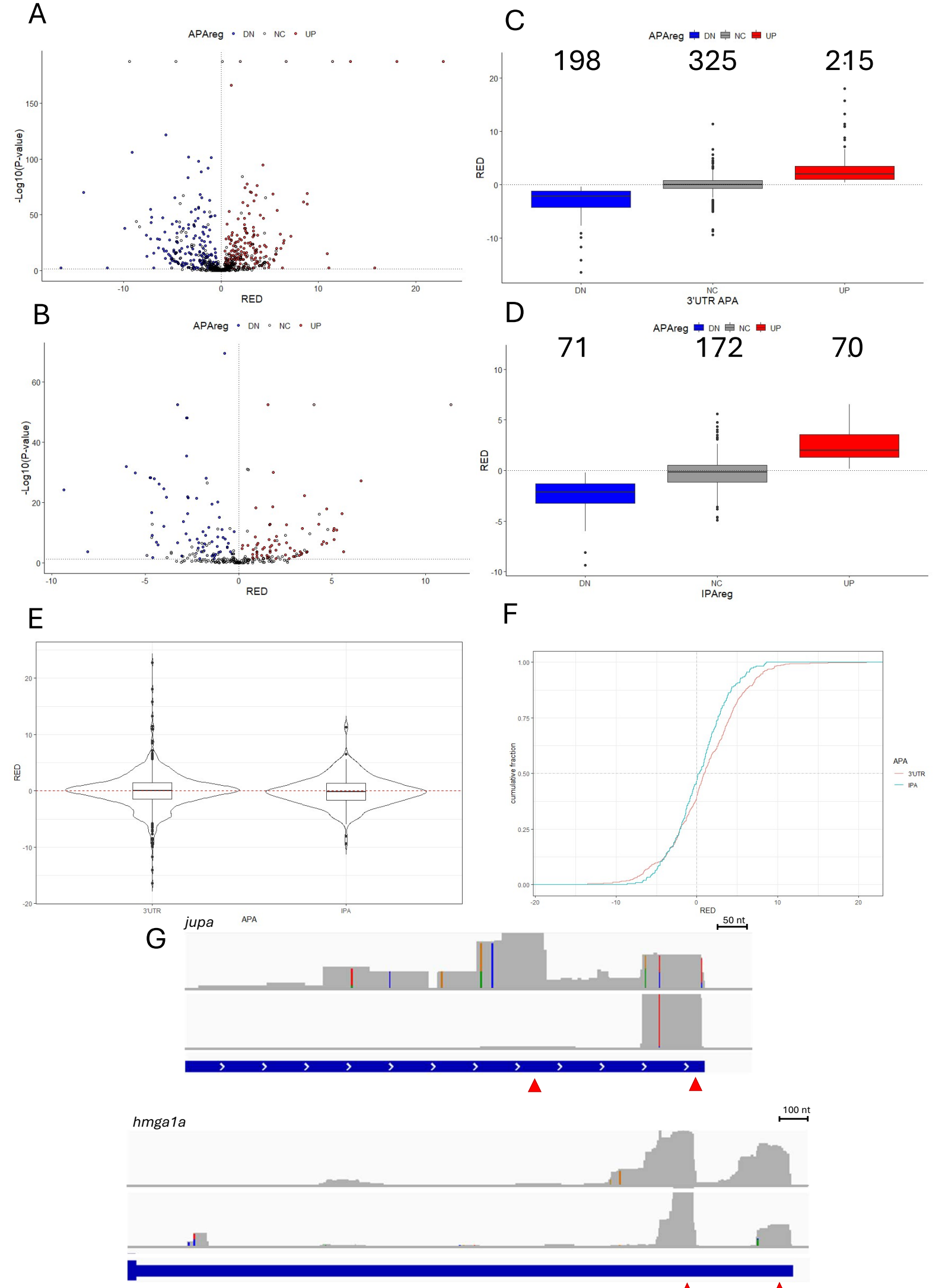

A
APAreg DN NC UP
-Log10(P-value)
RED
B
APAreg DN NC UP
-Log10(P-value)
RED
C
APAreg DN NC UP
198
325
215
RED
DN
NC
UP
3'UTR APA
D
APAreg DN NC UP
71
172
70
RED
DN
NC
UP
IPAreg
E
RED
3'UTR
IPA
APA
F
cumulative fraction
APA
3'UTR
IPA
RED
G
jupa
50 nt
hmga1a
100 nt

Figure 11

RNA processing with a focus on APA at 2 hpf vs 4 hpf. (A) Volcano plot of lengthened (in red) and shortened (in blue) genes. RED = Relative Expression Difference. (B) Box plot of APA change. Numbers represent the number of genes with a significant RED between the two stages. DN = Shortened; NC = Unchanged; UP = Lengthened. (C) (D) Volcano plot and box plot of genes switching from a 3'UTR PAS to an intronic PAS (UP) or the opposite (DN). NC = Unchanged. (E) Violin plot of APA expression. The landscape is more varied from 2 hpf vs 4 hpf compared to 0 vs 4 hpf. (F) Cumulative distribution function of both 3'UTR only processing vs IPA use. The trend is similar at in both sets. (G) Examples of lengthening from 2 hpf to 4 hpf (*cdc20*) and shortening (*ell2*) as well as PAS (red arrowhead) used for each isoform.

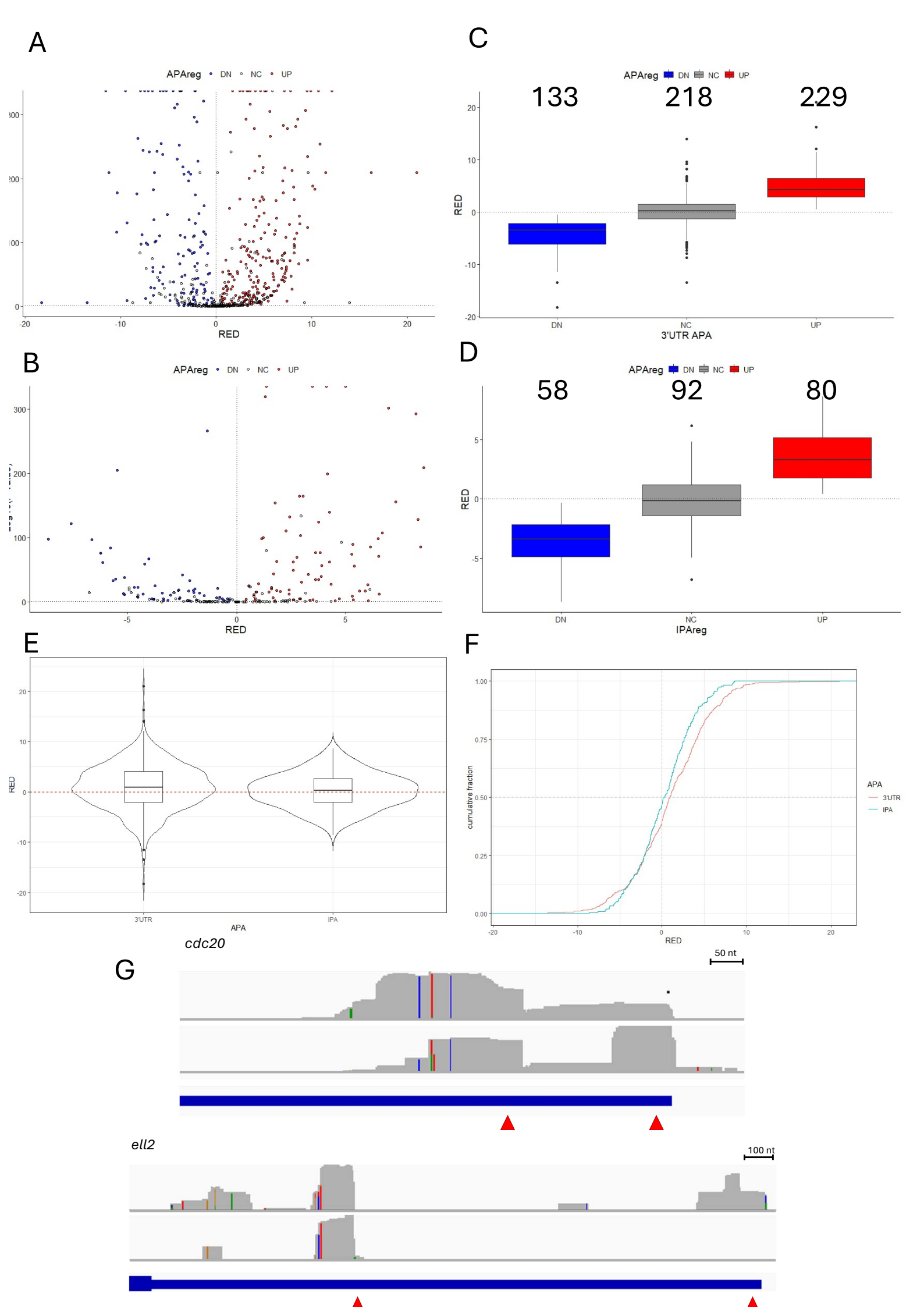
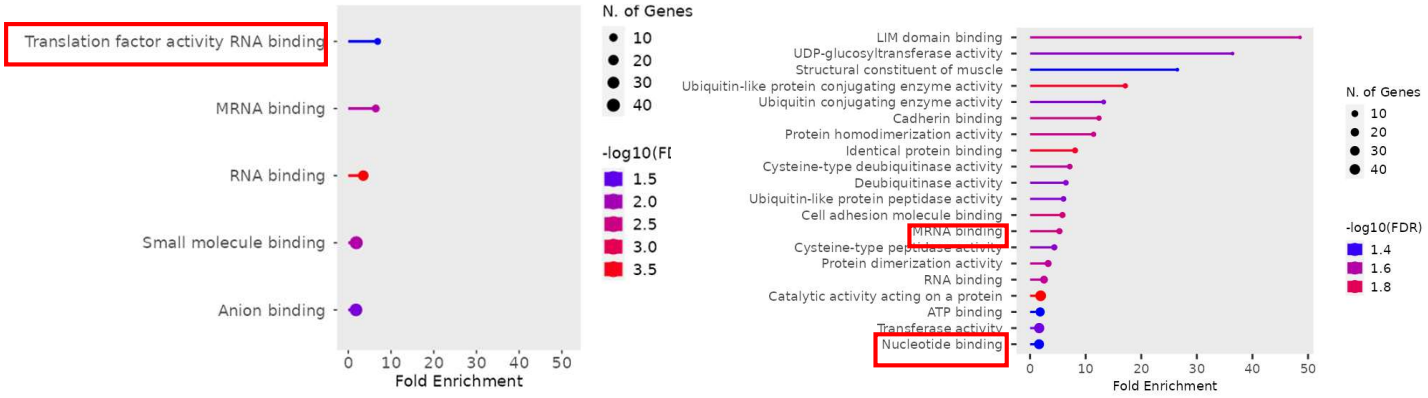

A
APAreg DN NC UP
RED
B
APAreg DN NC UP
RED
C
APAreg DN NC UP
133
218
229
RED
DN
NC
UP
3'UTR APA
D
APAreg DN NC UP
58
92
80
RED
DN
NC
UP
IPAreg
E
RED
3'UTR
IPA
APA
F
cumulative fraction
RED
APA
3'UTR
IPA
cdc20
G
50 nt
ell2
100 nt

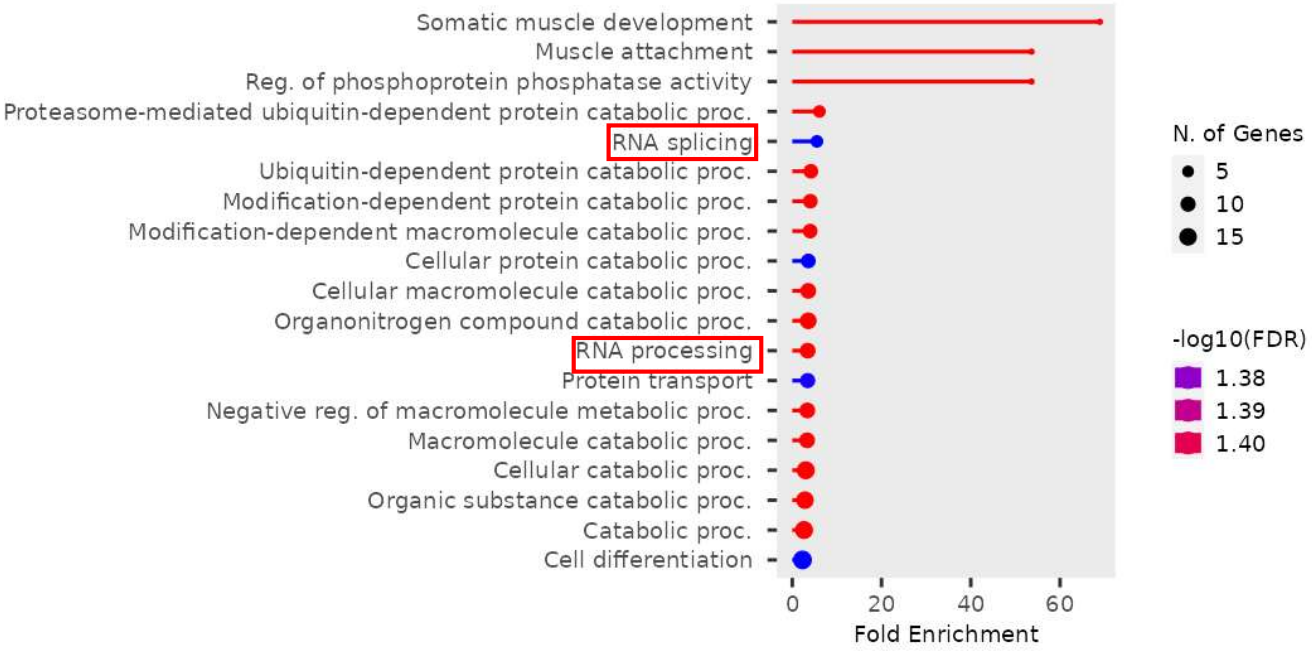
Figure 12

GO of APAlyzer genes. (A) Molecular binding of mRNA which underwent shortening (left) and lenghtening (right) by APA shift from 0 hpf to 2 hpf. (B) Cellular processes in which mRNA that were shortened (up) or lenghtened (down) by APA shift from 2 hpf to 4 hpf are involved. In red (A and B): genes involved in mRNA regulation

A


Translation factor activity RNA binding
MRNA binding
RNA binding
Small molecule binding
Anion binding
0 10 20 30 40 50
Fold Enrichment
N. of Genes
10
20
30
40
-log10(Fl
1.5
2.0
2.5
3.0
3.5
LIM domain binding
UDP-glucosyltransferase activity
Structural constituent of muscle
Ubiquitin-like protein conjugating enzyme activity
Ubiquitin conjugating enzyme activity
Cadherin binding
Protein homodimerization activity
Identical protein binding
Cysteine-type deubiquitinase activity
Deubiquitinase activity
Ubiquitin-like protein peptidase activity
Cell adhesion molecule binding
MRNA binding
Cysteine-type peptidase activity
Protein dimerization activity
RNA binding
Catalytic activity acting on a protein
ATP binding
Transferase activity
Nucleotide binding
0 10 20 30 40 50
Fold Enrichment
N. of Genes
10
20
30
40
-log10(FDR)
1.4
1.6
1.8

B


Somatic muscle development
Muscle attachment
Reg. of phosphoprotein phosphatase activity
Proteasome-mediated ubiquitin-dependent protein catabolic proc.
RNA splicing
Ubiquitin-dependent protein catabolic proc.
Modification-dependent protein catabolic proc.
Modification-dependent macromolecule catabolic proc.
Cellular protein catabolic proc.
Cellular macromolecule catabolic proc.
Organonitrogen compound catabolic proc.
RNA processing
Protein transport
Negative reg. of macromolecule metabolic proc.
Macromolecule catabolic proc.
Cellular catabolic proc.
Organic substance catabolic proc.
Catabolic proc.
Cell differentiation
0 20 40 60
Fold Enrichment
N. of Genes
5
10
15
-log10(FDR)
1.38
1.39
1.40

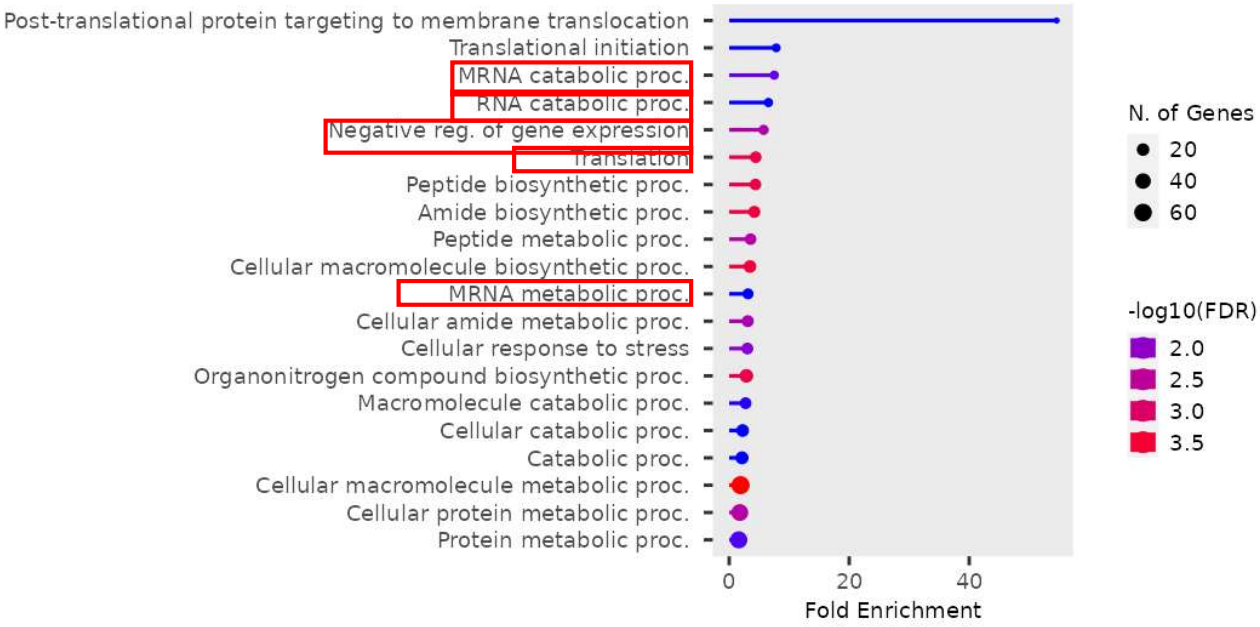
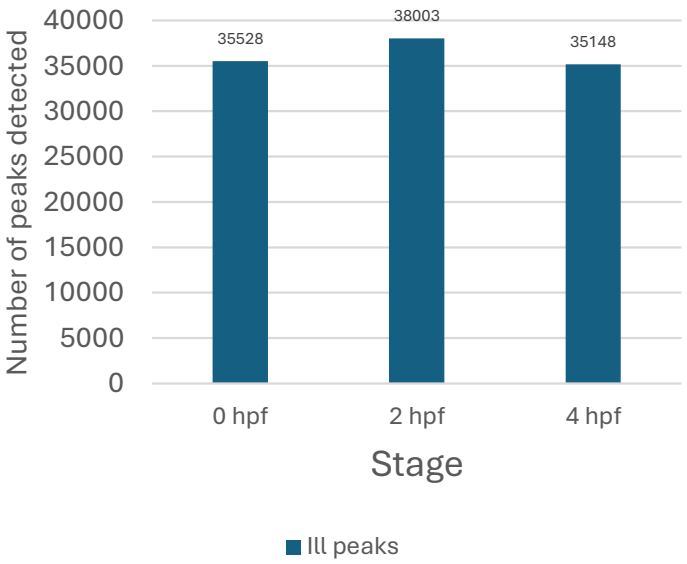

Post-translational protein targeting to membrane translocation
Translational initiation
MRNA catabolic proc.
RNA catabolic proc.
Negative reg. of gene expression
Translation
Peptide biosynthetic proc.
Amide biosynthetic proc.
Peptide metabolic proc.
Cellular macromolecule biosynthetic proc.
MRNA metabolic proc.
Cellular amide metabolic proc.
Cellular response to stress
Organonitrogen compound biosynthetic proc.
Macromolecule catabolic proc.
Cellular catabolic proc.
Catabolic proc.
Cellular macromolecule metabolic proc.
Cellular protein metabolic proc.
Protein metabolic proc.
0 20 40
Fold Enrichment
N. of Genes
20
40
60
-log10(FDR)
2.0
2.5
3.0
3.5

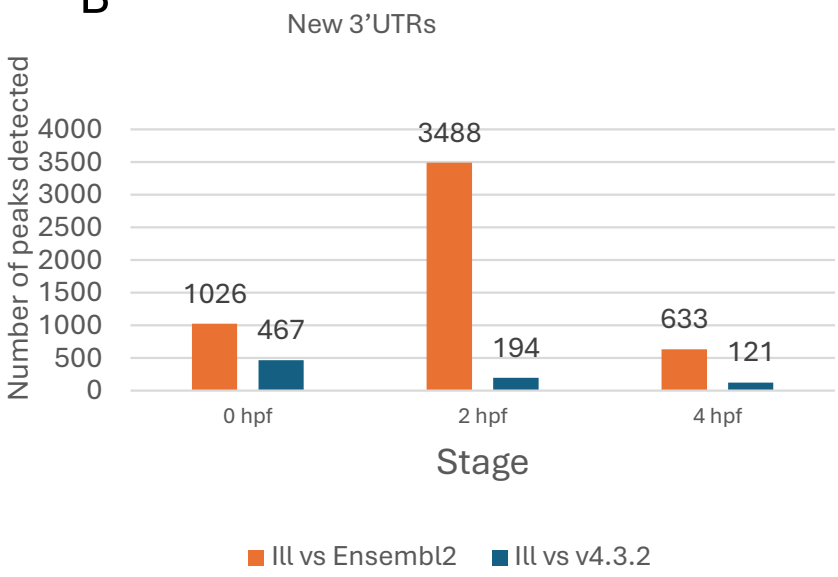
Figure 13

Peaks2utr analysis of the genome. (A) Number of peaks detected by peaks2utr. The numbers were similar between all stages hinting the gene expression to not be dissimilar. (B) New UTRs discovered by peaks2utr. Lawson and colleagues' data was also used as a reference for more accurate annotation. In order, the data ran was: Illumina against Ensembl, with a maximum distance of 3000 bases; Illumina against Lawson and colleagues' data with a maximum distance of 3000 bases. Ill vs Ensembl2 = This thesis' RNA-seq data vs the at-the-time most up to date Ensembl reference file. Ill vs v4.3.2 = This thesis' RNA-seq data vs the at-the-time most up to date Lawson and colleagues newly annotated reference file and Ensembl reference file. (C) Peak number per gene for 55 genes. For more than half of the genes, over 2 different peaks were detected, consistent with results in (A). (D) Example of a newly annotated UTR. This alternative UTR is only annotated as an exon in Ensembl. (Similar type to *wtn8a*, annotated in Ensembl).

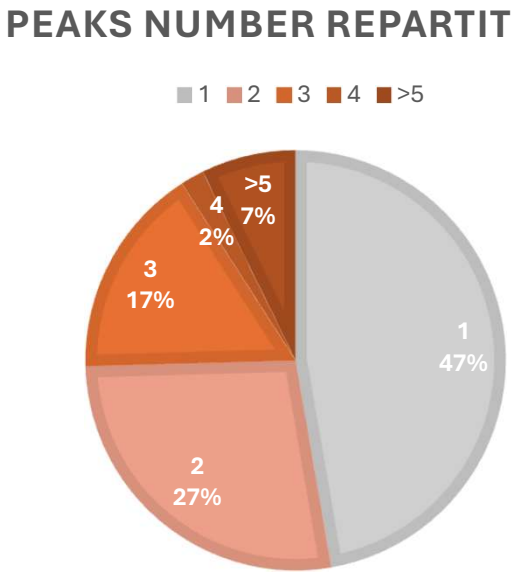
A



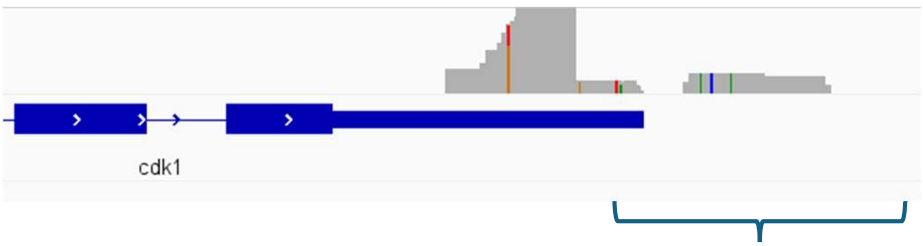
B



C



D

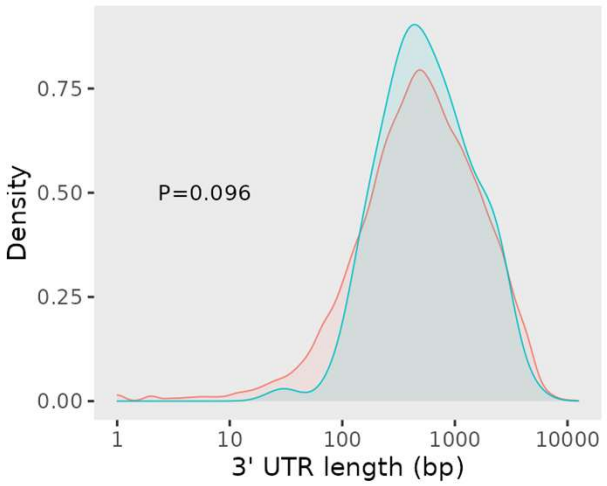


New 3'UTR

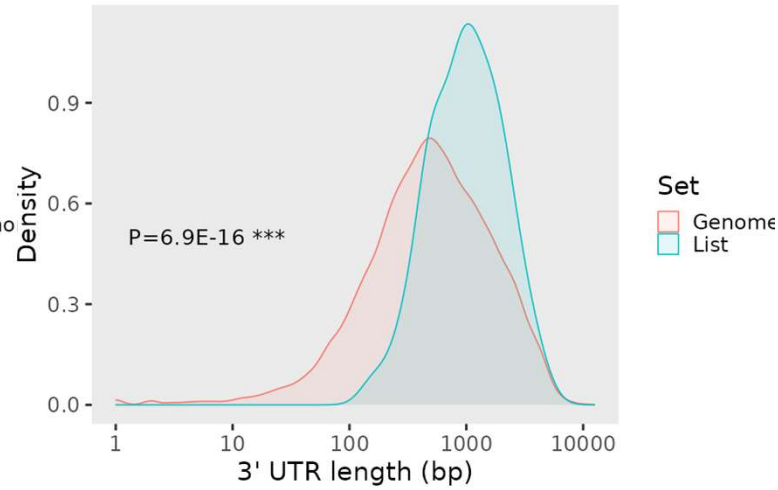
Figure 14

Gene ontology of class I vs class II genes. (A) 3'UTR length of class I genes (B) 3'UTR length of class II genes. The transcripts are on average longer than the average of zebrafish transcript. (C) Class I gene ontology for 100 genes. The genes strongly enriched are involved in, notably, transcription regulation. (D) KEGG of genes involved in transcription regulation. (E) Class II gene ontology for 100 genes. Many genes seem maternally inherited with some involved oocyte maturation. (F) Number of isoforms for class II genes compared to the average in zebrafish. The distribution confirms peaks2utr data.

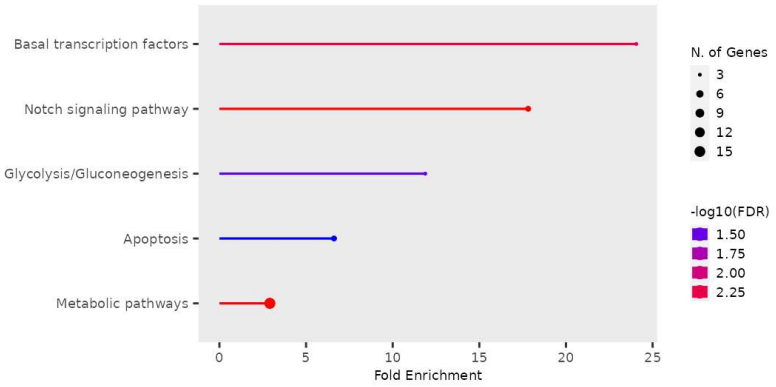
A


Density
0.75
0.50
0.25
0.00
P=0.096
1
10
100
1000
10000
3' UTR length (bp)
Set
Geno
List

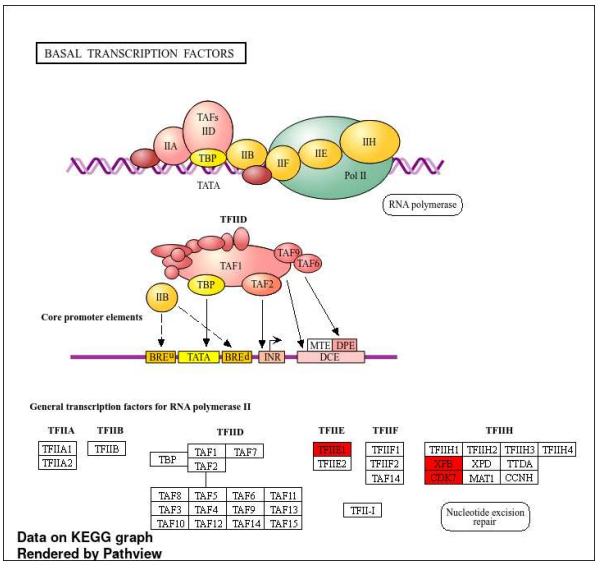
B


Density
0.9
0.6
0.3
0.0
P=6.9E-16 ***
1
10
100
1000
10000
3' UTR length (bp)
Set
Genome
List

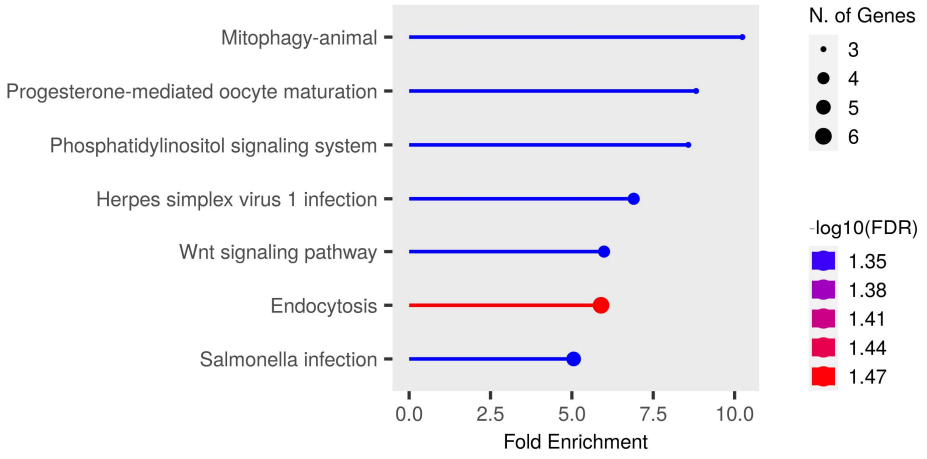
C


Basal transcription factors
Notch signaling pathway
Glycolysis/Gluconeogenesis
Apoptosis
Metabolic pathways
0
5
10
15
20
25
Fold Enrichment
N. of Genes
3
6
9
12
15
-log10(FDR)
1.50
1.75
2.00
2.25

D


BASAL TRANSCRIPTION FACTORS
TAFs
IID
IIA
TBP
IIB
IIF
IIE
IIH
Pol II
TATA
RNA polymerase
TFIID
TAF1
TAF9
TAF6
TBP
TAF2
IIB
Core promoter elements
BRE(d)
TATA
BRE(u)
INR
MTE
DPE
DCE
General transcription factors for RNA polymerase II
TFIIA
TFIIA1
TFIIA2
TFIIB
TFIIB
TFIID
TBP
TAF1
TAF2
TAF7
TAF8
TAF5
TAF6
TAF11
TAF3
TAF4
TAF9
TAF13
TAF10
TAF12
TAF14
TAF15
TFIIE
TFIIE1
TFIIE2
TFIIF
TFIIF1
TFIIF2
TAF14
TFII-I
TFIIH
TFIIH1
TFIIH2
TFIIH3
TFIIH4
XPB
XPD
TTDA
CDK7
MAT1
CCNH
Nucleotide excision repair
Data on KEGG graph
Rendered by Pathview

E


Mitophagy-animal
Progesterone-mediated oocyte maturation
Phosphatidylinositol signaling system
Herpes simplex virus 1 infection
Wnt signaling pathway
Endocytosis
Salmonella infection
0.0
2.5
5.0
7.5
10.0
Fold Enrichment
N. of Genes
3
4
5
6
-log10(FDR)
1.35
1.38
1.41
1.44
1.47

F

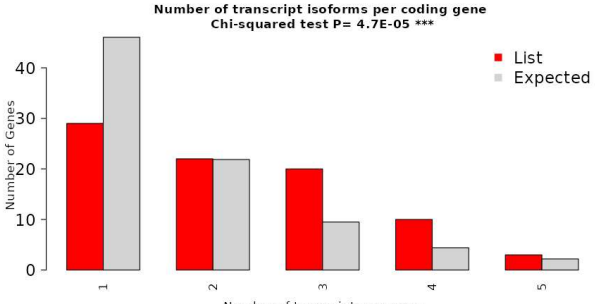
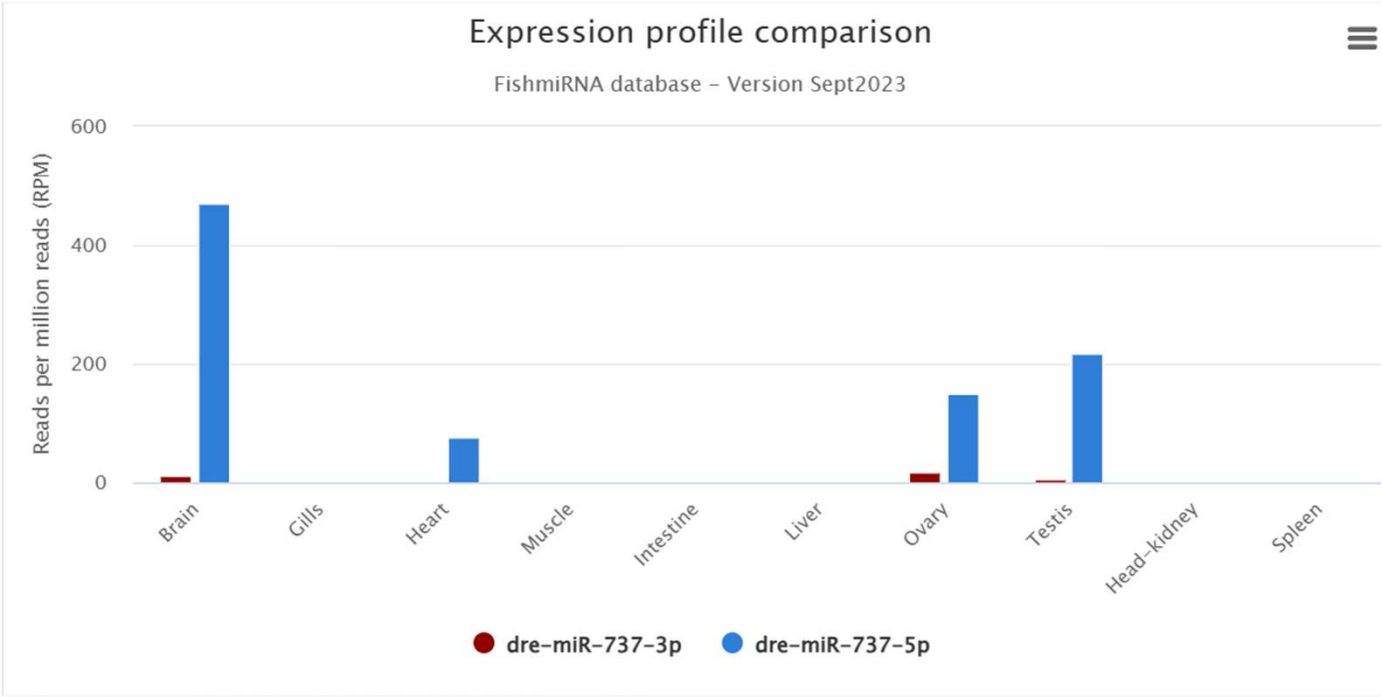

Number of transcript isoforms per coding gene
Chi-squared test P= 4.7E-05 ***
List
Expected
Number of Genes
40
30
20
10
0
1
2
3
4
5

Figure 15

miRNA expression in 1 hpf eggs. (A) Potential seed deleted in short *pou5f3* is expressed in zebrafish ovary. (B) Seed potentially impacted by mRNA remodelling after shortening. It is strongly expressed in the ovary.

A


Expression profile comparison
FishmiRNA database – Version Sept2023
Reads per million reads (RPM)
600
400
200
0
Brain
Gills
Heart
Muscle
Intestine
Liver
Ovary
Testis
Head-kidney
Spleen
dre-miR-737-3p
dre-miR-737-5p

B

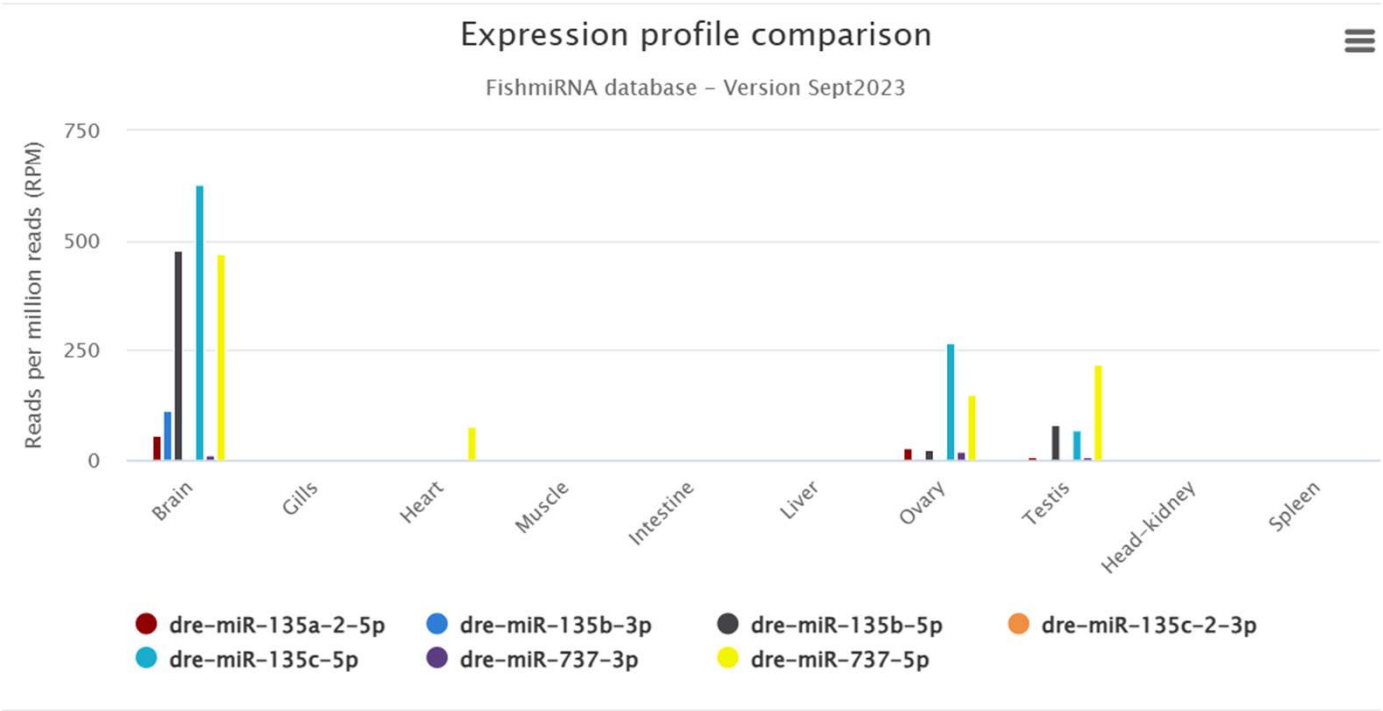
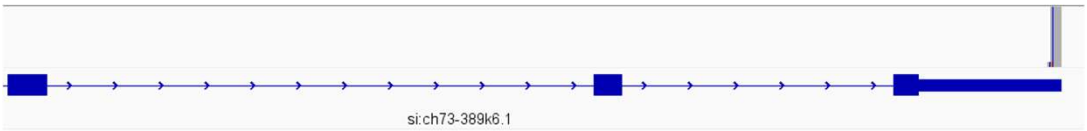
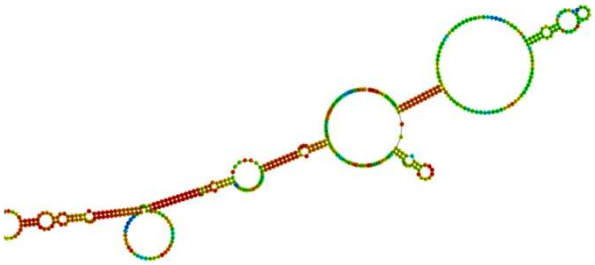

Expression profile comparison
FishmiRNA database – Version Sept2023
Reads per million reads (RPM)
750
500
250
0
Brain
Gills
Heart
Muscle
Intestine
Liver
Ovary
Testis
Head-kidney
Spleen
dre-miR-135a-2-5p
dre-miR-135b-3p
dre-miR-135b-5p
dre-miR-135c-2-3p
dre-miR-135c-5p
dre-miR-737-3p
dre-miR-737-5p

Figure 16

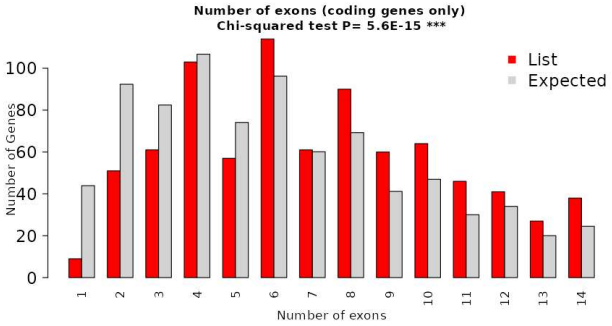
miRNA expression in 1 hpf eggs. (A) (Up) Most expressed small RNAs picked up by miRNA-sequencing. Rlf show different isoforms hitting toward an unreferenced cluster. (down) 3D simulation of the “3-UTR-annotated” small RNA. Pin-like structure and instability are reminiscent of that of pre-miRNA. (B) GO of mRNA predicted to bind to dre-miR-21 using Targetscanfish (version 6.2) and selecting ovary found mRNAs.

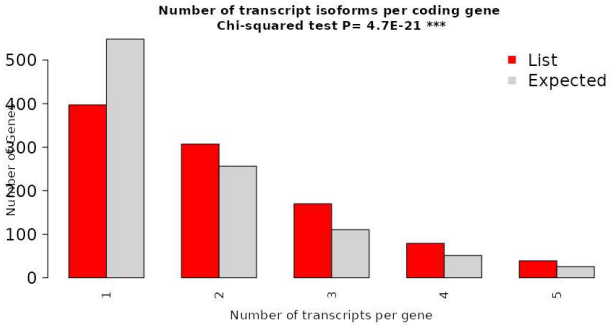
A

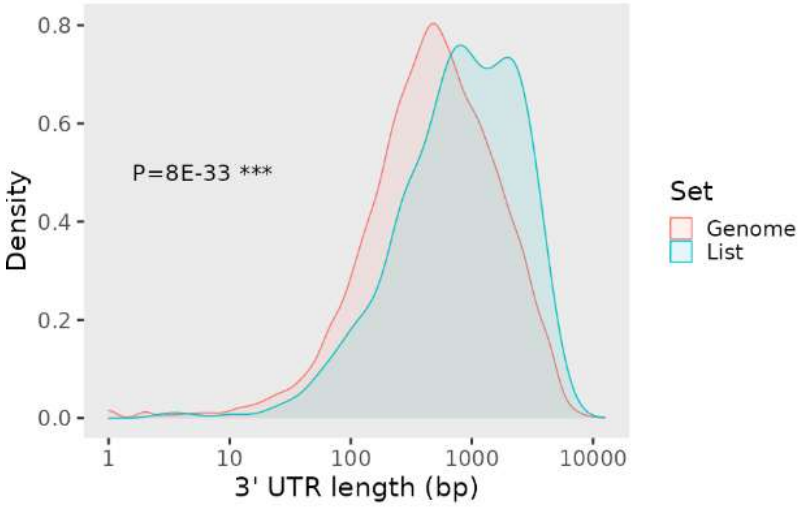

si:ch73-389k6.1



B


Number of exons (coding genes only)
Chi-squared test P= 5.6E-15 ***
List
Expected
Number of Genes
Number of exons


Number of transcript isoforms per coding gene
Chi-squared test P= 4.7E-21 ***
List
Expected
Number of Genes
Number of transcripts per gene


P=8E-33 ***
Density
3' UTR length (bp)
Set
Genome
List

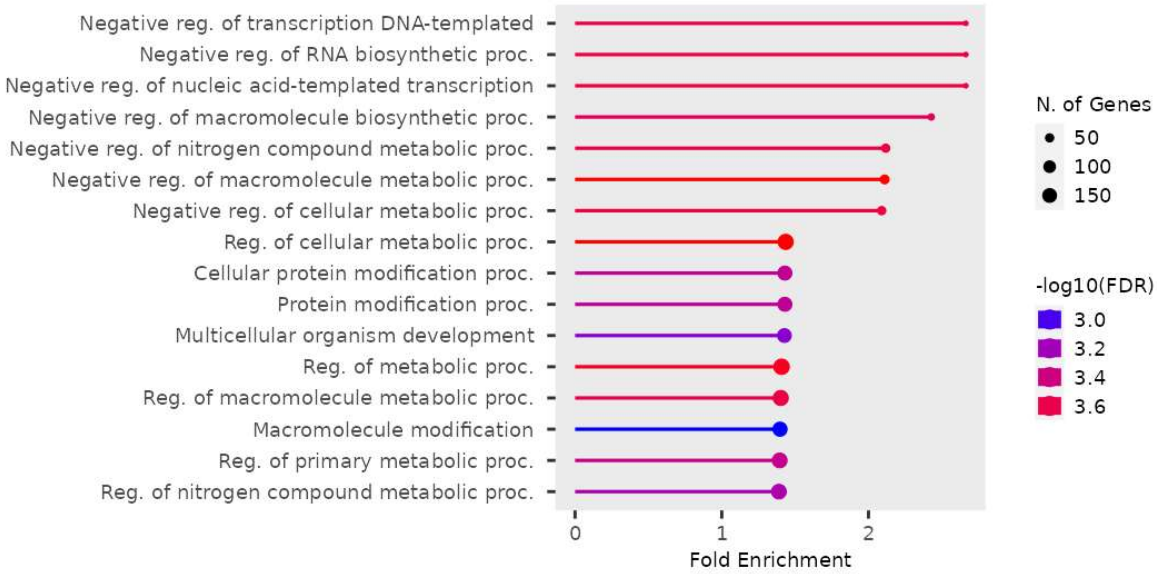
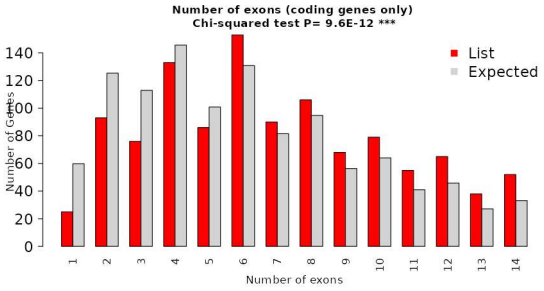
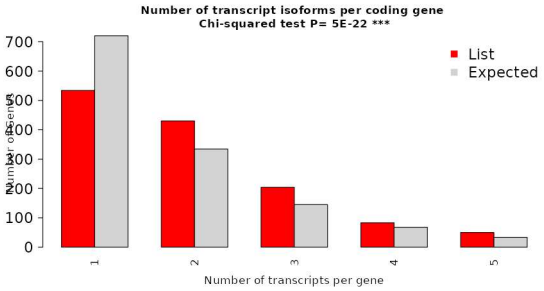

Negative reg. of transcription DNA-templated
Negative reg. of RNA biosynthetic proc.
Negative reg. of nucleic acid-templated transcription
Negative reg. of macromolecule biosynthetic proc.
Negative reg. of nitrogen compound metabolic proc.
Negative reg. of macromolecule metabolic proc.
Negative reg. of cellular metabolic proc.
Reg. of cellular metabolic proc.
Cellular protein modification proc.
Protein modification proc.
Multicellular organism development
Reg. of metabolic proc.
Reg. of macromolecule metabolic proc.
Macromolecule modification
Reg. of primary metabolic proc.
Reg. of nitrogen compound metabolic proc.
Fold Enrichment
N. of Genes
50
100
150
-log10(FDR)
3.0
3.2
3.4
3.6

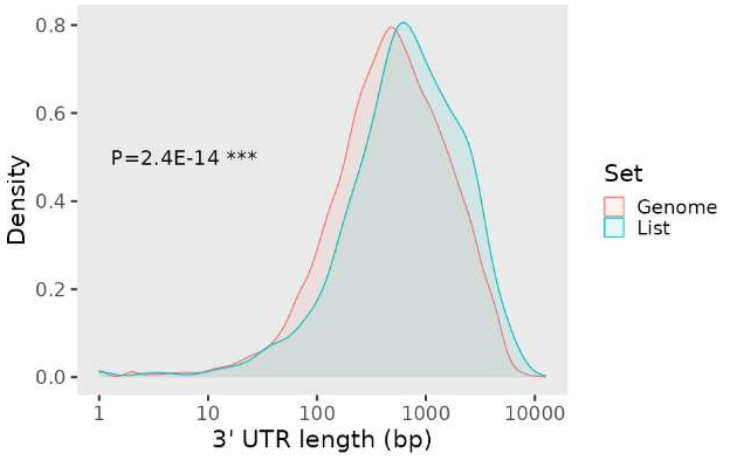
Figure 17

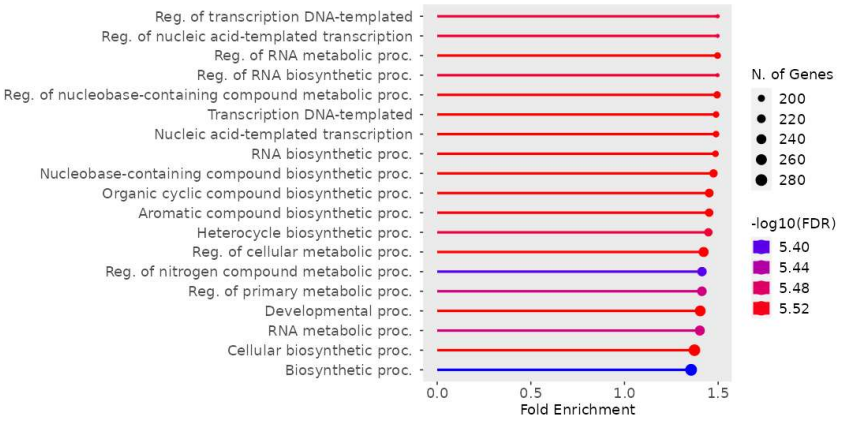
miRNA expression in 1 hpf eggs. (A) GO of mRNA predicted to bind to dre-miR-22 using Targetscanfish (version 6.2) and selecting ovary found mRNAs. (B) GO of mRNA predicted to bind to miR-181using Targetscanfish (version 6.2) and selecting ovary found mRNAs.

A

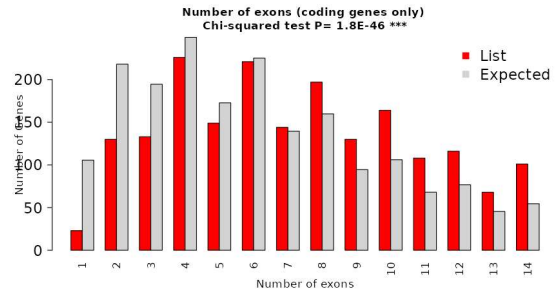

Number of exons (coding genes only)
Chi-squared test P= 9.6E-12 ***
List
Expected
Number of Genes
Number of exons

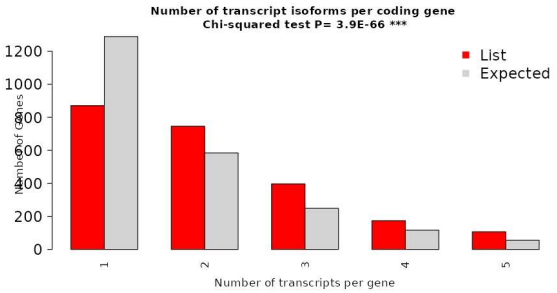

Number of transcript isoforms per coding gene
Chi-squared test P= 5E-22 ***
List
Expected
Number of Genes
Number of transcripts per gene

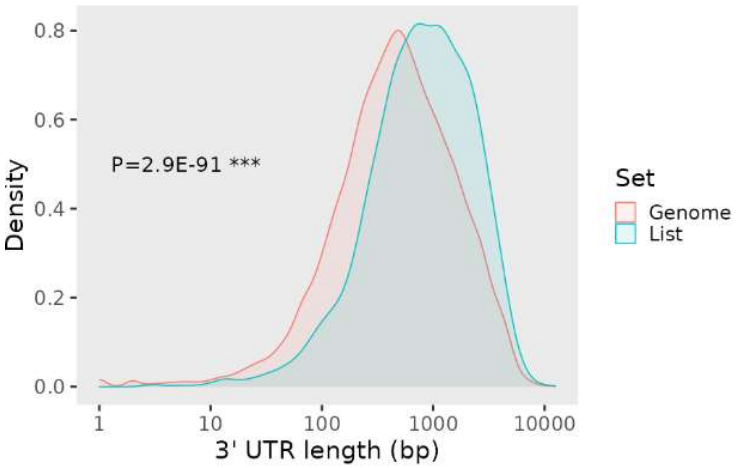

P=2.4E-14 ***
Density
3' UTR length (bp)
Set
Genome
List


Reg. of transcription DNA-templated
Reg. of nucleic acid-templated transcription
Reg. of RNA metabolic proc.
Reg. of RNA biosynthetic proc.
Reg. of nucleobase-containing compound metabolic proc.
Transcription DNA-templated
Nucleic acid-templated transcription
RNA biosynthetic proc.
Nucleobase-containing compound biosynthetic proc.
Organic cyclic compound biosynthetic proc.
Aromatic compound biosynthetic proc.
Heterocycle biosynthetic proc.
Reg. of cellular metabolic proc.
Reg. of nitrogen compound metabolic proc.
Reg. of primary metabolic proc.
Developmental proc.
RNA metabolic proc.
Cellular biosynthetic proc.
Biosynthetic proc.
Fold Enrichment
N. of Genes
-log10(FDR)

B


Number of exons (coding genes only)
Chi-squared test P= 1.8E-46 ***
List
Expected
Number of Genes
Number of exons


Number of transcript isoforms per coding gene
Chi-squared test P= 3.9E-66 ***
List
Expected
Number of Genes
Number of transcripts per gene


P=2.9E-91 ***
Density
3' UTR length (bp)
Set
Genome
List

F

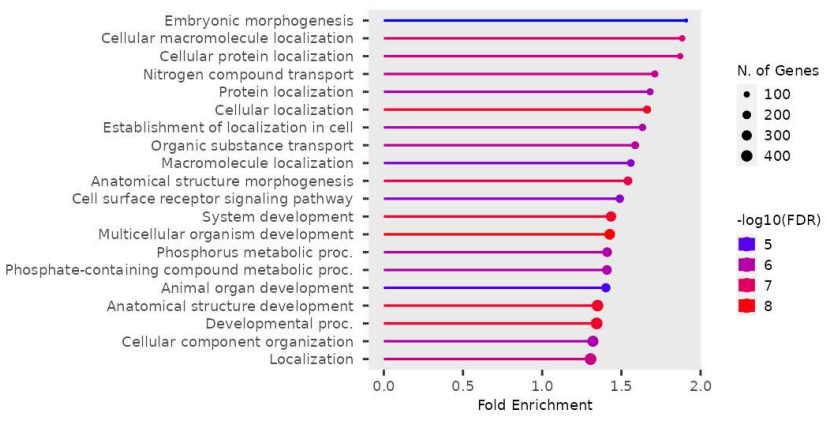
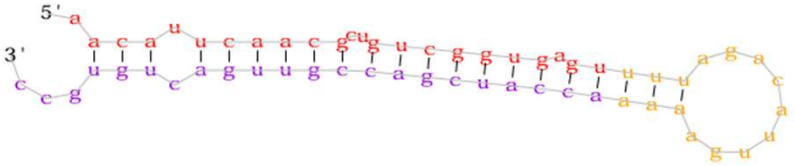

Embryonic morphogenesis
Cellular macromolecule localization
Cellular protein localization
Nitrogen compound transport
Protein localization
Cellular localization
Establishment of localization in cell
Organic substance transport
Macromolecule localization
Anatomical structure morphogenesis
Cell surface receptor signaling pathway
System development
Multicellular organism development
Phosphorus metabolic proc.
Phosphate-containing compound metabolic proc.
Animal organ development
Anatomical structure development
Developmental proc.
Cellular component organization
Localization
Fold Enrichment
N. of Genes
-log10(FDR)

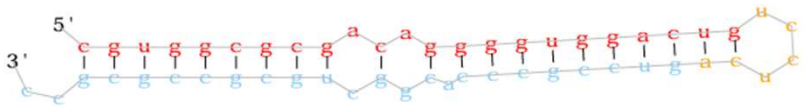
Figure 18

Novel miRNAs at 1 hpf. Hairpin structure of the four novel miRNAs with the best confidence score.

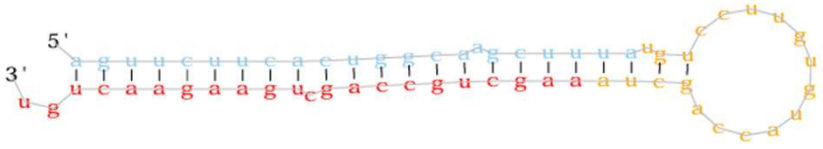
Provisional ID	:	2_2725
Score total	:	31.9
Score for star read(s)	:	3.9
Score for read counts	:	25.1
Score for mfe	:	1.3
Score for randfold	:	1.6
Score for cons. seed	:	
Total read count	:	61
Mature read count	:	53
Loop read count	:	0
Star read count	:	8



Provisional ID	:	4_3469
Score total	:	3.3
Score for star read(s)	:	-1.3
Score for read counts	:	0
Score for mfe	:	3
Score for randfold	:	1.6
Score for cons. seed	:	
Total read count	:	283
Mature read count	:	283
Loop read count	:	0
Star read count	:	0



Provisional ID	:	15_1054
Score total	:	2.5
Score for star read(s)	:	-1.3
Score for read counts	:	0
Score for mfe	:	2.2
Score for randfold	:	1.6
Score for cons. seed	:	
Total read count	:	146
Mature read count	:	146
Loop read count	:	0
Star read count	:	0



Provisional ID	:	10_875
Score total	:	2.5
Score for star read(s)	:	-1.3
Score for read counts	:	0
Score for mfe	:	2.2
Score for randfold	:	1.6
Score for cons. seed	:	
Total read count	:	158
Mature read count	:	158
Loop read count	:	0
Star read count	:	0

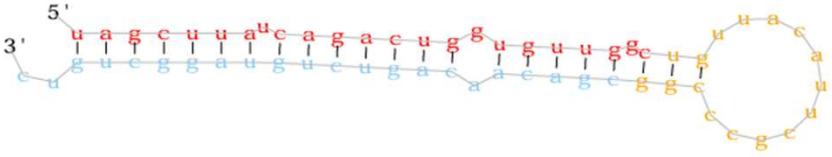
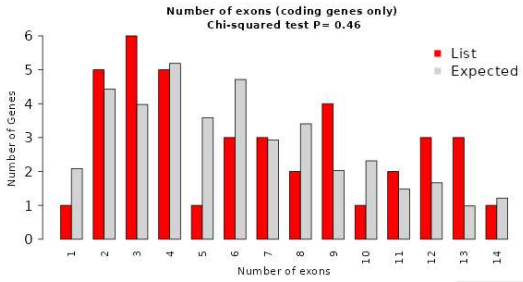
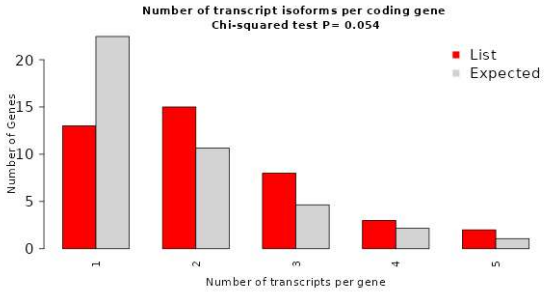


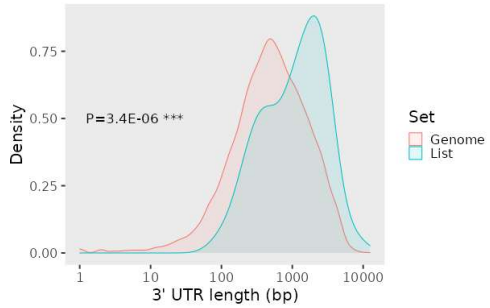
Figure 19

Novel miRNAs at 1 hpf. (A-D) GO of the four novel miRNAs with the best confidence score’s target. All tend to have targets that are long, coding, with multiple isoforms and and a long 3'UTR.

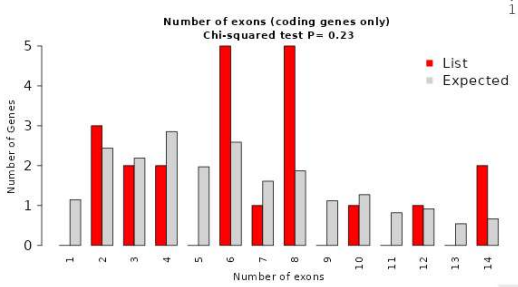
A

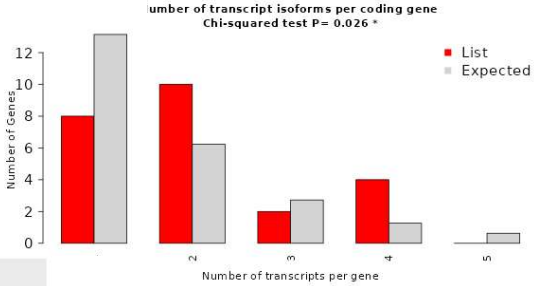

Number of exons (coding genes only)
Chi-squared test P= 0.46
List
Expected
Number of Genes
Number of exons

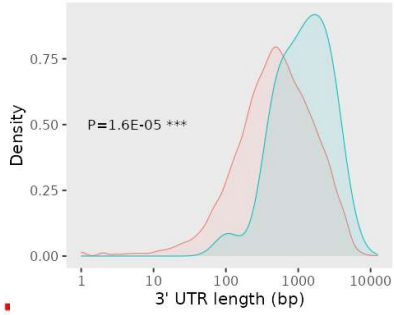

Number of transcript isoforms per coding gene
Chi-squared test P= 0.054
List
Expected
Number of Genes
Number of transcripts per gene


P=3.4E-06 ***
Density
3' UTR length (bp)
Set
Genome
List

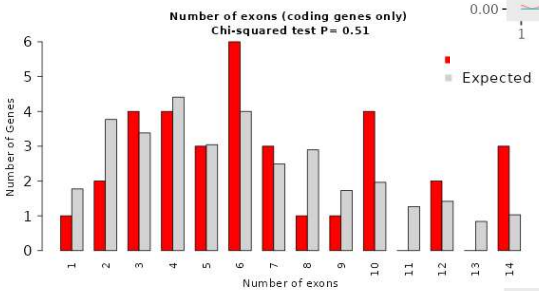
B

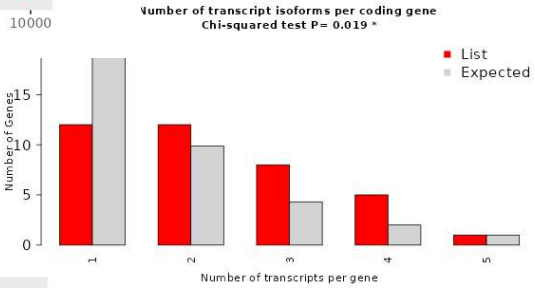

Number of exons (coding genes only)
Chi-squared test P= 0.23
List
Expected
Number of Genes
Number of exons

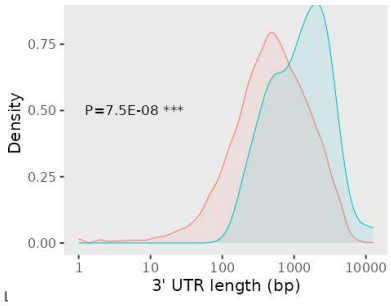

umber of transcript isoforms per coding gene
Chi-squared test P= 0.026 *
List
Expected
Number of Genes
Number of transcripts per gene


P=1.6E-05 ***
Density
3' UTR length (bp)
Set
Genon
List

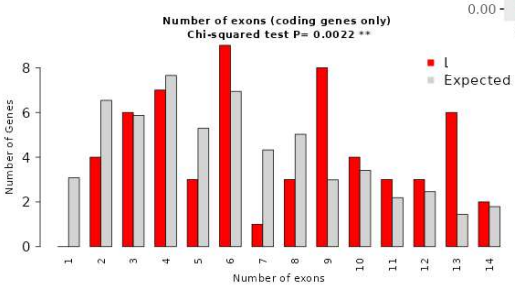
C

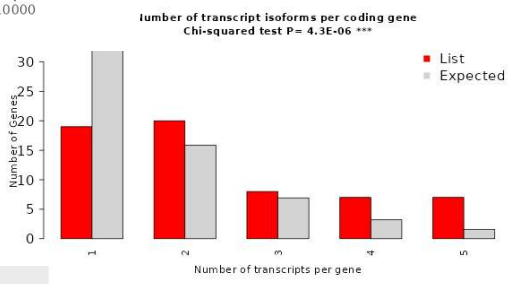

Number of exons (coding genes only)
Chi-squared test P= 0.51
Expected
Number of Genes
Number of exons


Number of transcript isoforms per coding gene
Chi-squared test P= 0.019 *
List
Expected
Number of Genes
Number of transcripts per gene


P=7.5E-08 ***
Density
3' UTR length (bp)
Set
Genome
List

D


Number of exons (coding genes only)
Chi-squared test P= 0.0022 **
Expected
Number of Genes
Number of exons


Number of transcript isoforms per coding gene
Chi-squared test P= 4.3E-06 ***
List
Expected
Number of Genes
Number of transcripts per gene

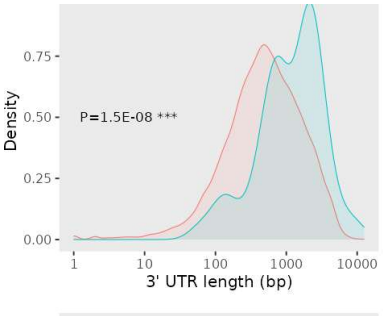

P=1.5E-08 ***
Density
3' UTR length (bp)
Set
Genome
List

Figure 20

Novel miRNAs at 1 hpf. (A) and (B) Blast of novel miRNA binding to the 72 last bases of the 3'end of *pou5f3*. In red: site 1; in yellow: site 2

A

```
Query   13   TGACTGT   19
             |||||||
Sbjct   72   TGACTGT   66

Query   3    CATCGAC   9
             |||||||
Sbjct   61   CATCGAC   67
```

B

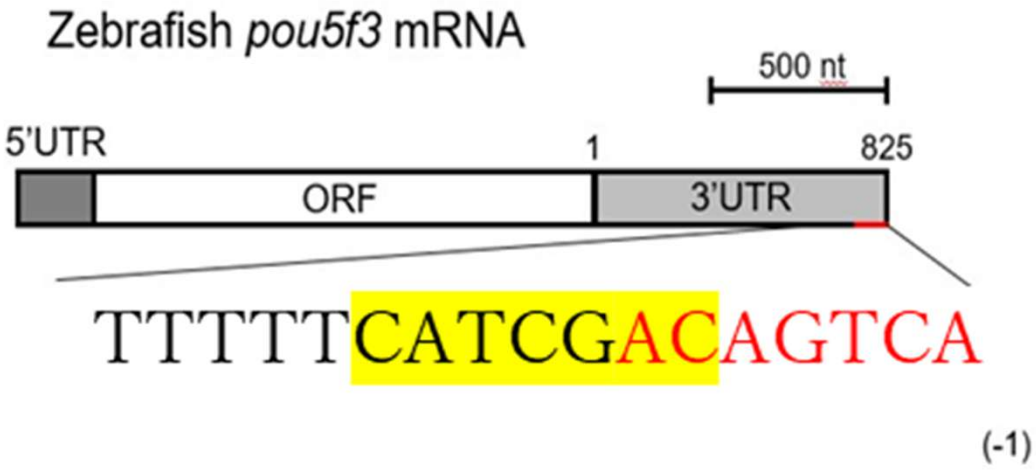
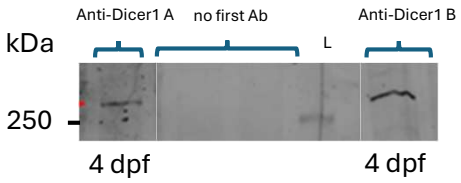


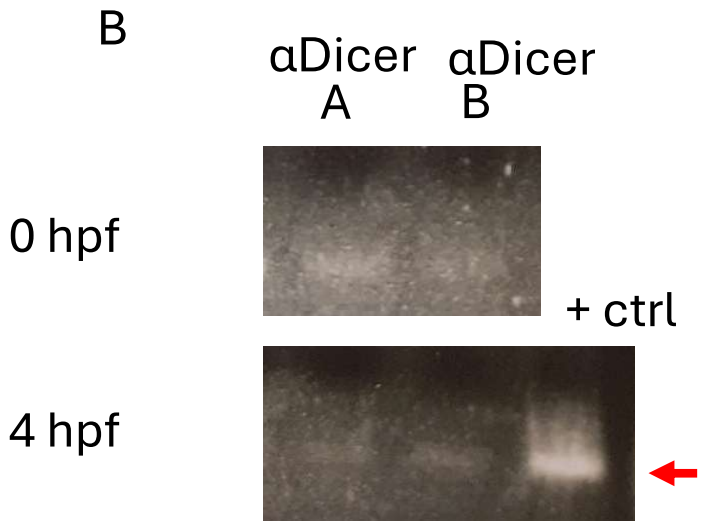
Figure 21

Dicer is expressed from the earliest stages in zebrafish. (A) Western blotting of Dicer1 using antibodies generated using zebrafish Dicer1. (B) Immunoprecipitation of Dicer1. *pou5f3* could not be found to be bound at 0 hpf nor 4 hpf. (C) Localisation of Dicer1 at 0, 2, 3, 4 and 6 hpf. Dicer appears to create clusters from 4 hpf onwards. It also localises in some nuclei (arrow). (D) Confocal microscope pictures of Dicer1. Dicer1 expression increases until 4 hpf to decrease at 6 hpf.

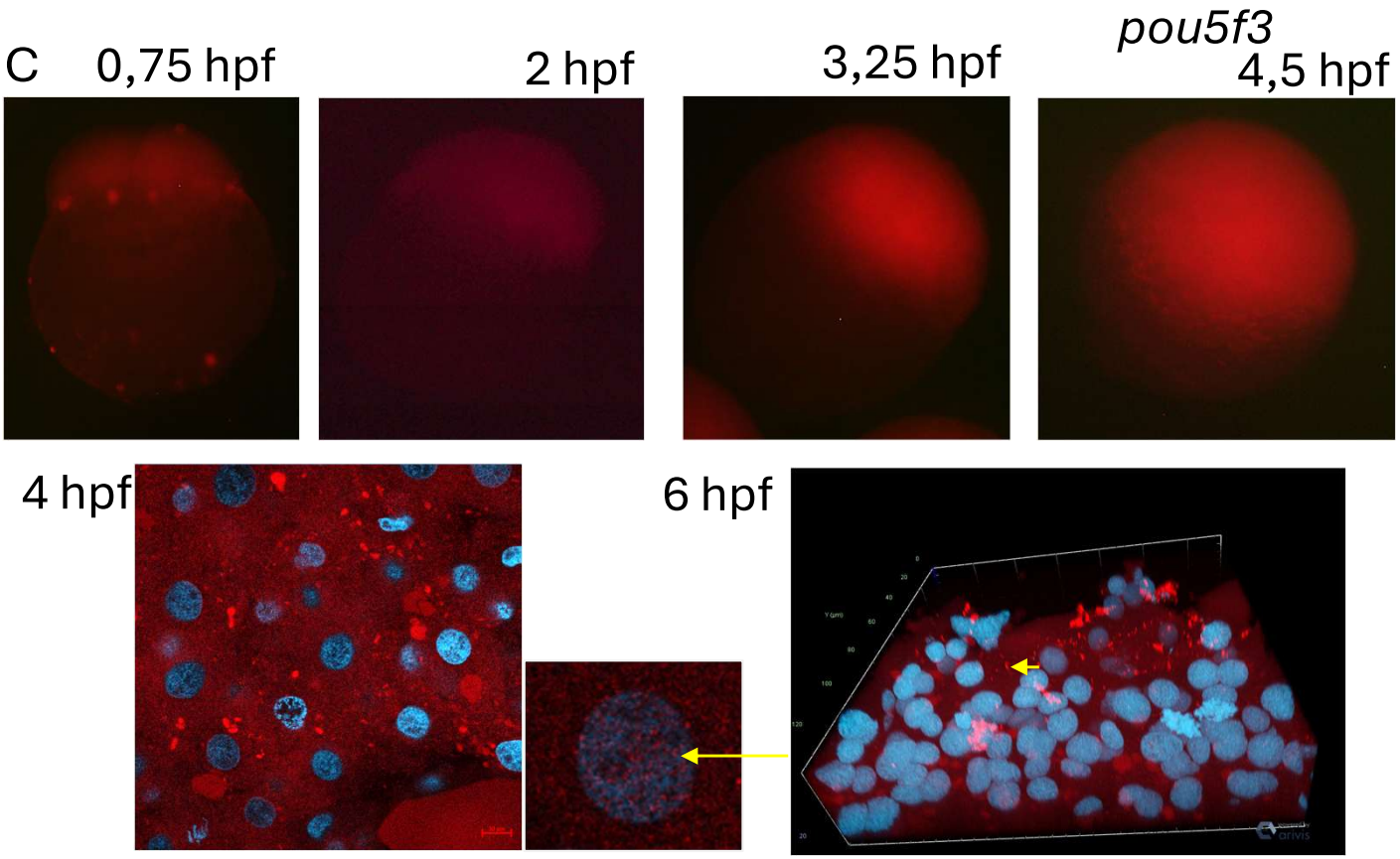
A


Anti-Dicer1 A
no first Ab
Anti-Dicer1 B
kDa
L
250
4 dpf
4 dpf

B


αDicer A
αDicer B
0 hpf
+ ctrl
4 hpf

C


0,75 hpf
2 hpf
3,25 hpf
pou5f3
4,5 hpf
4 hpf
6 hpf

D

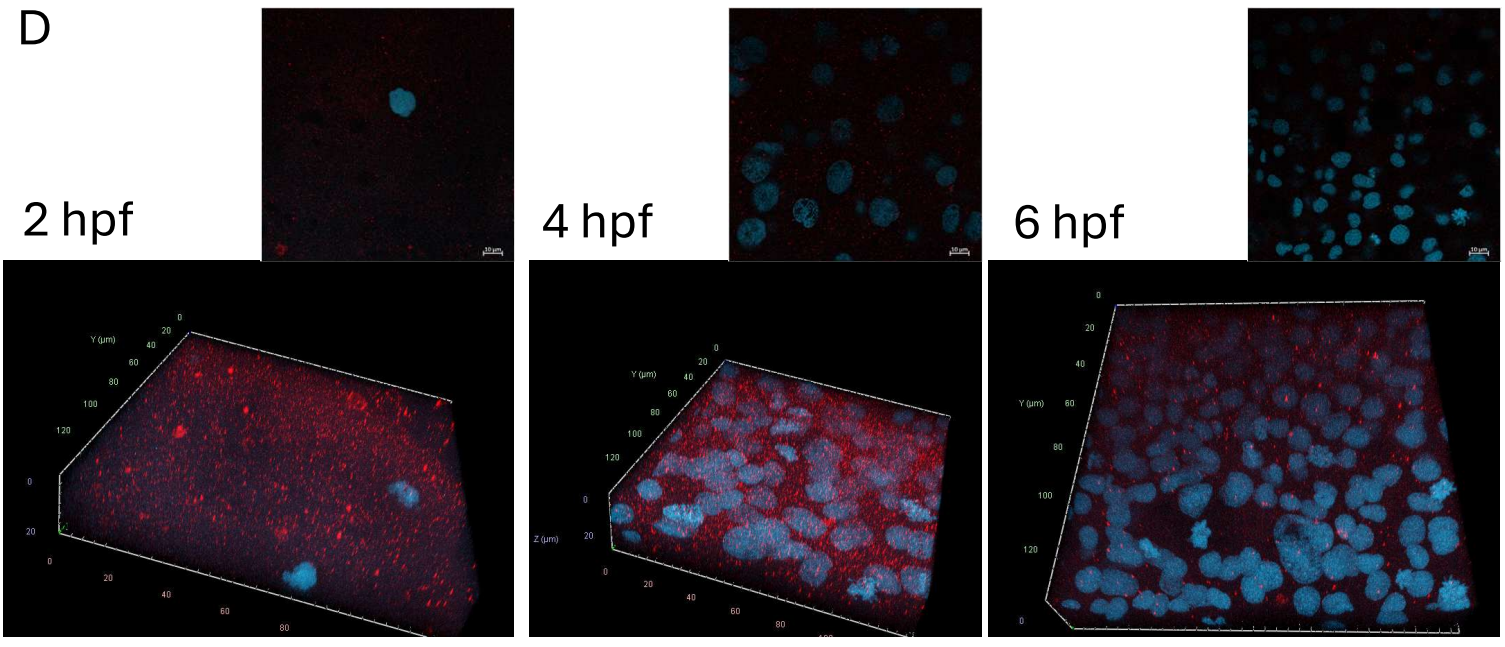
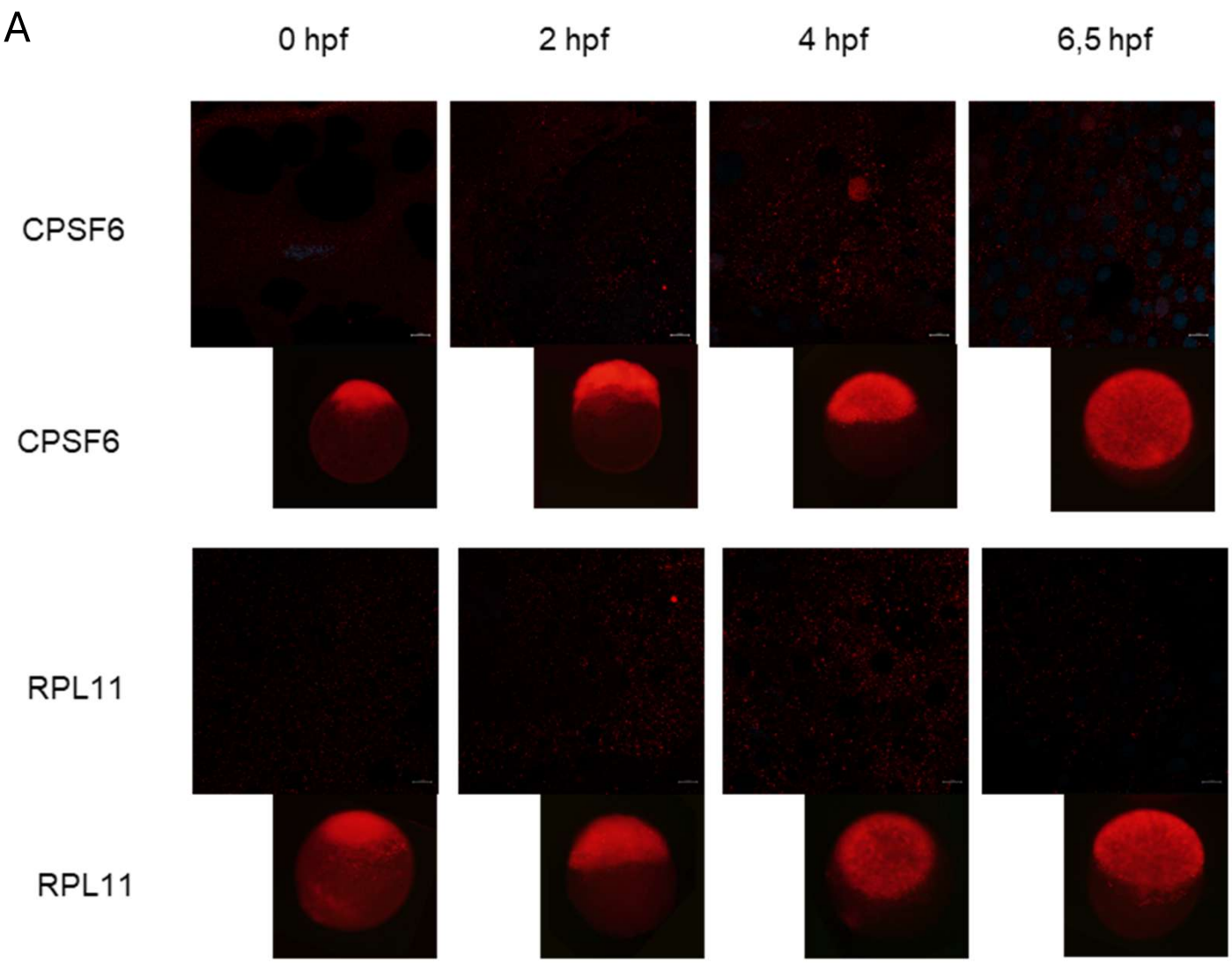
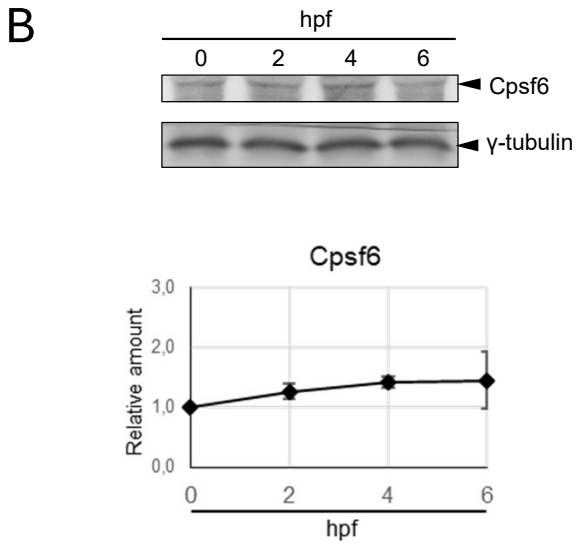
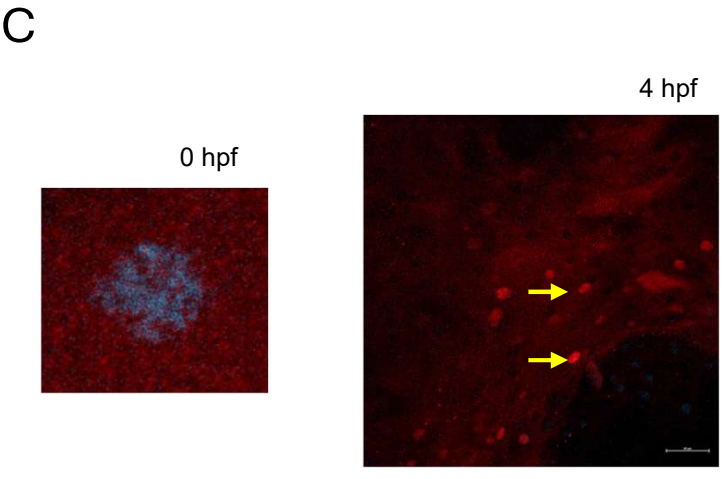

2 hpf
4 hpf
6 hpf

Figure 22

CPSF6 expression location changes with isoform change. (A) Overall expression expression of CPSF6 under fluorescent light with a stereo microscope and confocal microscope. CPSF6 seem to be overall most present at 4 hpf. (B) Western blotting of CPSF6. No significant changes could be observed from 0 to 6 hpf. (C) Nuclear views of CPSF6 at 2 hpf vs 4 hpf. Although CPSF6 already localised in the nucleus at 2 hpf, its relative amount is drastically different compared to that observable at 4 hpf. (D) Z-stack views of CPSF6 expression from 0 hpf to 6 hpf. CPSF6 creates big conglomerates at 4 hpf in the cytoplasm but also fully fills the nucleus at certain mitotic stages but not others.


A
0 hpf
2 hpf
4 hpf
6,5 hpf
CPSF6
CPSF6
RPL11
RPL11


B
hpf
0
2
4
6
Cpsf6
γ-tubulin
Cpsf6
Relative amount
3,0
2,0
1,0
0,0
0
2
4
6
hpf


C
0 hpf
4 hpf

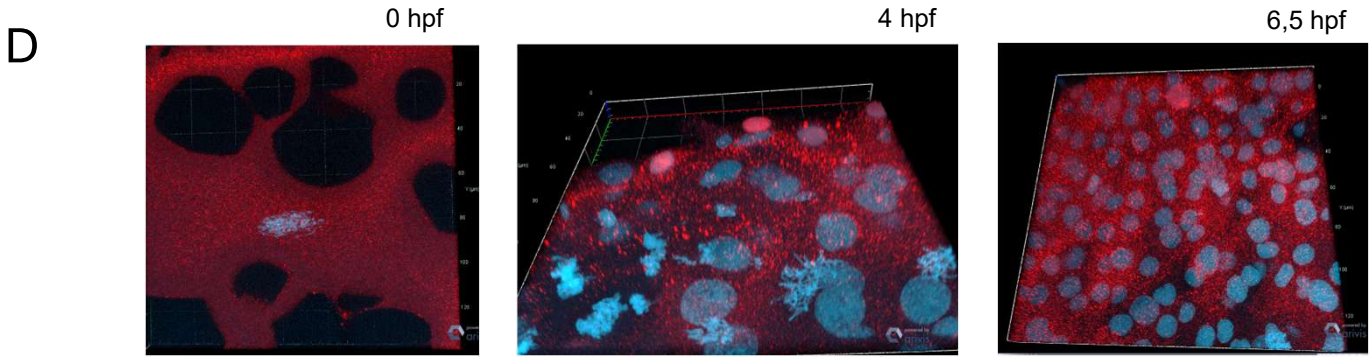
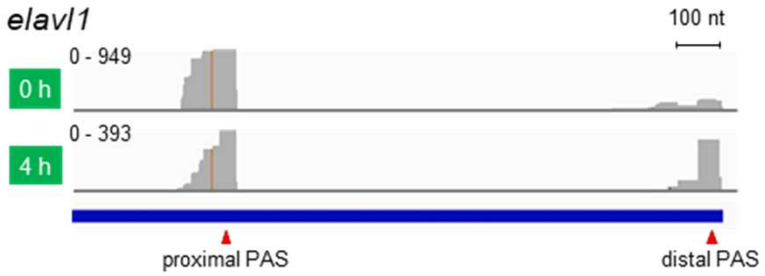

D
0 hpf
4 hpf
6,5 hpf

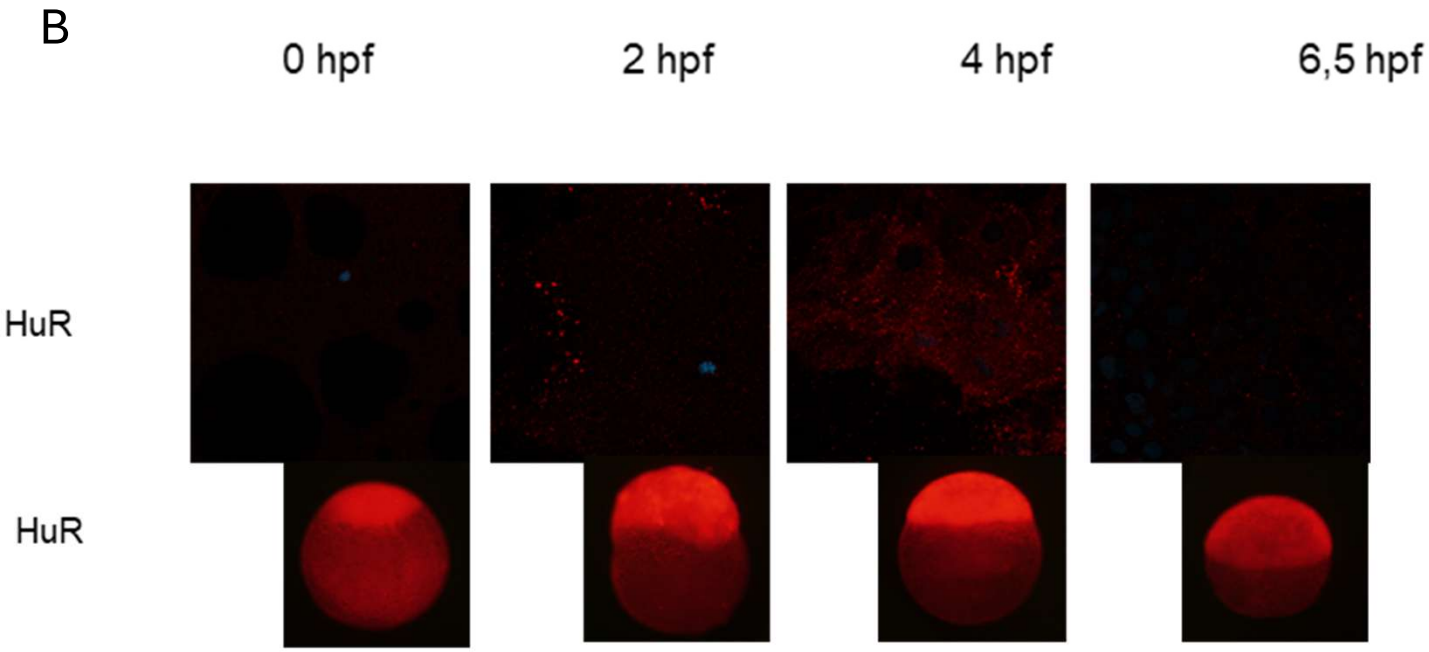
Figure 23

HuR expression is on the rise in the early stages of zebrafish development. (A) IGV view of *elavl1* shortening from 0 hpf to 4 hpf (B) Immunostaining of HuR under both a stereo and laser confocal microscope. HuR spatial expression pattern is consistent all throughout the stages with a gradual raise from 0 to 4 hpf. (C) Western blotting of HuR. Expression is significantly higher at 4 hpf compared to 0 hpf.

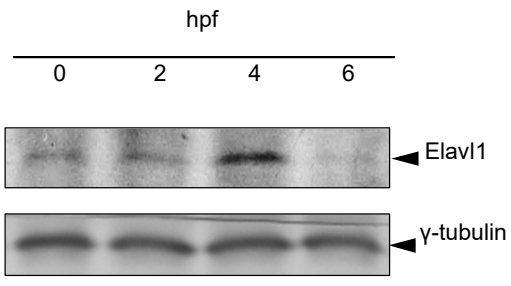
A


elavl1
100 nt
0 - 949
0 h
0 - 393
4 h
proximal PAS
distal PAS

B


0 hpf
2 hpf
4 hpf
6,5 hpf
HuR
HuR

C


hpf
0
2
4
6
Elavl1
γ-tubulin

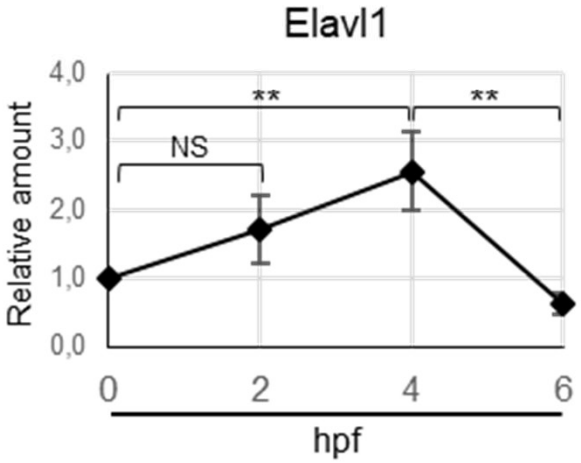
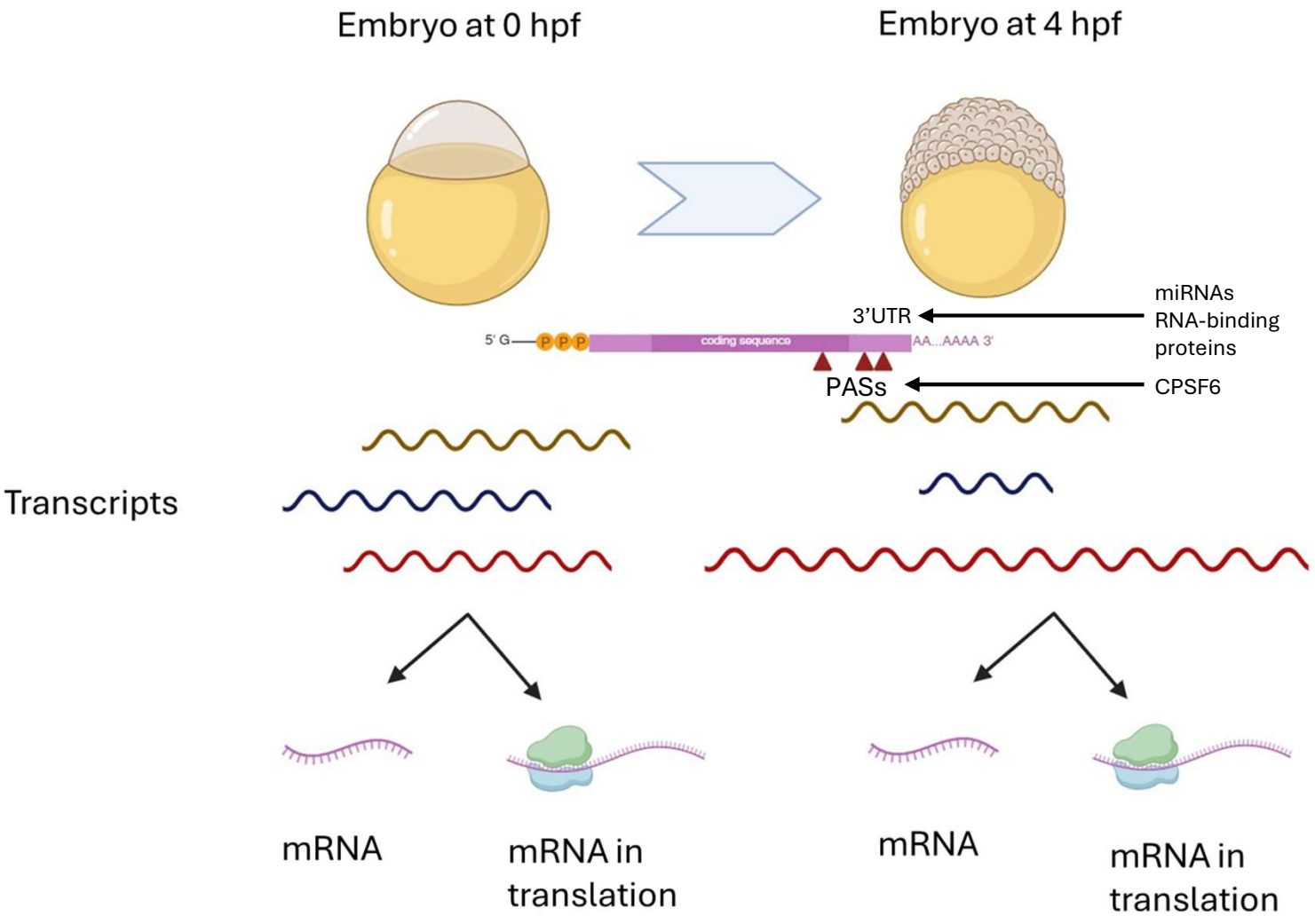

Elavl1
Relative amount
4,0
3,0
2,0
1,0
0,0
**
**
NS
0
2
4
6
hpf

Figure 24

Graphical summary of the thesis.

Red arrows: PAS

mRNA processing events from top to bottom: unchanged (in brown), shortening (in blue) and lengthening (in red) activating or not mRNA translation.


Embryo at 0 hpf
Embryo at 4 hpf
3'UTR
miRNAs
RNA-binding
proteins
5' G
coding sequence
AA...AAAA 3'
PASs
CPSF6
Transcripts
mRNA
mRNA in
translation
mRNA
mRNA in
translation